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Defining the Roles of p300/CBP (CREB Binding Protein) and S5a in p53 Polyubiquitination, Degradation and DNA Damage Responses: A Dissertation

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Defining the roles of p300/CBP (CREB Binding Protein) and S5a in p53 polyubiquitination, degradation and DNA damage responses

A Dissertation Presented

By

Dingding Shi

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January 8th, 2010

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Defining the roles of p300/CBP (CREB Binding Protein) and S5a in p53 polyubiquitination, degradation and DNA damage responses

Abstract

p53, known as the “guardian of the genome”, is the most well-characterized tumor suppressor gene. The central role of p53 is to prevent genome instability. p53 is the central node in an incredibly elaborate genome defense network for receiving various input stress signals and controlling diverse cellular responses. The final output of this network is determined not only by the p53 protein itself, but also by other p53 cooperating proteins.

p300 and CBP (CREB-Binding Protein) act as multifunctional regulators of p53 via acetylase and ubiquitin ligase activities. Prior work in vitro has shown that the N-terminal 595 aa of p300 encode both generic ubiquitin ligase (E3) and p53-directed E4 functions. Analysis of p300 or CBP-deficient cells revealed that both coactivators were required for endogenous p53 polyubiquitination and the normally rapid turnover of p53 in unstressed cells. Unexpectedly, p300/CBP ubiquitin ligase activities were absent in nuclear extracts and exclusively cytoplasmic. In the nucleus, CBP and p300 exhibited differential regulation of p53 gene target expression, C-terminal acetylation, and biologic response after DNA damage. p300 activated, and CBP repressed, PUMA

expression, correlating with activating acetylation of p53 C-terminal lysines by p300, and a repressive acetylation of p53 lysine-320 induced by CBP. Consistent with their gene expression effects, CBP deficiency augmented, and p300 deficiency blocked, apoptosis after doxorubicin treatment. Subcellular compartmentalization of p300/CBP's ubiquitination and transcription activities reconciles seemingly opposed functions—cytoplasmic p300/CBP E4 activities ubiquitinate and destabilize p53, while nuclear p300/CBP direct p53 acetylation, target gene activation, and biological outcome after genotoxic stress.

p53 is a prominent tumor suppressor gene and it is mutated in more than 50% of human tumors. Reactivation of endogenous p53 is one therapeutic avenue to stop cancer cell growth. In this thesis, we have identified S5as a critical regulator of p53 degradation and activity. S5a is a non-ATPase subunit in the 19S regulatory particle of the 26S proteasome. Our preliminary data indicates that S5a is required for p53 instability and is a negative regulator of p53 tranactivation. As a negative regulator of p53, S5a may therefore also represent a new target for cancer drug development against tumors that specifically maintain wild type p53.

Chapter 1: Introduction

1.1 The discovery of p53 as a tumor suppressor gene

p53 is the most prominent and intensively studied tumor suppressor gene over the last thirty years of cancer research. In 1979, David Lane and Arnold Levine simultaneously discovered a 53000 dalton protein in the immunoprecipitated complex of the simian virus 40 (SV-40) T antigen protein (91, 99). This protein was later named p53 and designated as an oncogene. By using the p53 cDNA clone, together with the Ras oncogene, three groups successfully either immortalized cells or fully transformed cells (38, 72, 119). This first cloned p53 cDNA was actually a dominant-negative allele, with a mutation at codon 135(valine to alanine), which led "p53" to act as an oncogene. The Levine group subsequently showed that the wild type p53 protein can actively inhibit oncogene transformation (47), and the Vogelstein lab found the p53 gene frequently mutated in human colon carcinomas (115), consistent with native p53 acting as a tumor suppressor gene. Following this pioneering work, many more lines of evidence pointed to the tumor suppressor function of p53 in human carcinogenesis. For example, the p53 mutations were found in very diversified human tumor types (94), and the Li-Fraumeni syndrome of multiple cancers at an early age was discovered to be due to inherited p53 mutations(69).

1.2. p53 is a sequence-specific transcription factor

The sequences of the p53 locus from more than 16,000 tumor samples have been collected and analyzed. Strikingly, 97% of the mutations fall into the DNA binding domain of p53 (59, 117). This sequence specific DNA binding domain plays an undoubtedly crucial role in the tumor suppressor function of p53. Besides the evolutionarily conserved DNA binding domain between amino acid 108 to 298, p53 also contains a transactivation domain in the N-terminus which is highly post-translationally modified. Therefore p53 is able to bind and transactivate its target genes. The consensus sequence for p53 binding has two copies of the inverted pentameric sequence PuPuPuC(A/T)(T/A)GPyPyPy separated by a 0-13 base pair long intervening fragment. There are 300-1600 predicted binding sites for p53 throughout the human genome, according to microarray and computational analysis (15, 64). So far, about 40 genes have been shown to contain p53 responsive elements and have been experimentally demonstrated to be transcriptional targets of p53. As a transcription factor that regulates a broad range of target genes falling into different functional groups, p53 is able to coordinate diverse cellular responses to a variety of cell stress factors.

1.3. p53 responds to a broad range of cellular stresses.

Sitting at the nexus of a complicated network, p53 senses and integrates diverse signals and converts them into highly coordinated gene expression patterns. The transactivation activity of p53 is kept silent or extremely low in wild type cells, and is activated when cells are exposed to stresses like DNA damage, oncogene activation, hypoxia, etc. Once activated, depending on the stress type and the microenvironment, p53 selectively turns on its transcriptional target genes that function in cell cycle arrest, DNA damage repair, or apoptosis, to generate different cellular adaptive responses. It is not clear how the information is integrated to selectively target p53 to different sets of at target gene promoters. P53 chooses to either pause cell growth to allow time for DNA repair, or will kill cells bearing non-repairable lesions. Since the active p53 can inhibit cell growth or even kill cells, a stringent regulatory mechanism is required to prevent the errant activation of p53.

1.4 p53 function is controlled by post-translational modifications

p53 modifications, including phosphorylation, ubiquitination, acetylation, methylation, sumoylation and neddylation, make up a very complex epigenetic code that intricately modulates p53 functions. Among all these modifications, ubiquitination, phosphorylation and acetylation are the most extensively studied types of modification on the p53 protein. They are involved in the regulation of all three steps required for p53 activation: 1) p53 stabilization 2) DNA binding 3) transcriptional activation.

1.4.1 Ubiquitination

p53 is almost non-detectable in normal cells because of a very short half life, ranging from 5 to 30 min in different cell types. The degradation of p53 is ubiquitination dependent and mediated by the proteasome, as is the degradation of more than 70% of the other proteins in cells. Ubiquitination requires the sequential action of three enzymes (121). E1 activating enzyme forms a thioester between the C-terminal glycine of ubiquitin and its own active site cysteine(121). Ubiquitin is then transferred to the active site cysteine of an E2 conjugating enzyme (121). An E3 ubiquitin ligase then facilitates transfer of ubiquitin to the protein substrate, resulting in rapid proteasome degradation (121). The E3 ubiquitin ligase, unlike E1 and E2, is specific to the protein substrate. E3's cooperate with E1 and E2 to catalyze the conjugation of poly-ubiquitin chains onto protein substrates so the ubiquitin conjugated substrate can be recognized by the 26 S proteasome for proteolysis. E3's provide substrate selectivity through a specific substrate recognition domain on itself, or via other cofactors in the E3 ubiquitin complex.

E6-AP (E6-Associated Protein) was the first identified p53 E3 ubiquitin ligase. E6AP targets p53 within a complex with high-risk human papillomavirus E6 proteins(66). Since E6-AP negatively regulates p53 activity, high-risk HPV infected cells are released from cell cycle arrest allowing viral genome replication(127). E6-AP contains a HECT (Homolog of E6-AP C Terminus) domain involved in catalyzing

ubiquitination.

Later, MDM2 was discovered as the principal physiologic E3 ubiquitin ligase of p53. The RING finger structure in the C-terminal end of MDM2 encodes a ubiquitin ligase activity by bringing together the E2 active site and the substrate's acceptor lysines (30, 41). That MDM2 plays the role as a key negative regulator of p53 is underscored by mouse genetic studies (27). The MDM2 null mouse is lethal due to hyperactive p53 induced apoptosis, and the lethality can be rescued by concurrent p53 deletion(74). Interestingly, the p53 protein level is elevated but still degradable in the MDM2-/- cells, which suggests that other E3 ubiquitin ligases of p53 might exist. Arf-BP1 (Arf Binding Protein 1), COP1, Pirh2 and synoviolin have also been characterized as E3 ubiquitin ligases of p53 in various contexts (20, 32, 93, 152, 156). Their physiological significance, however, remains to be determined.

1.4.2 Phosphorylation

P53 phosphorylation is the first critical step in the current model of p53 activation. The multiple N-terminal phosphorylation sites on p53 have been reported to be immediately stimulated by ATM/ ATR/DNA-PK and ChK1/ ChK2 after DNA damage or after other type of stresses (52, 130). The phosphorylation at Ser15 (mouse Ser18) and Ser20 (mouse Ser23) has been proposed to induce a conformational change to dissociate p53 from MDM2 (19, 131). Phosphorylated p53 becomes stabilized due to inhibited MDM2 mediated degradation, while further

increasing the binding affinity with p300/CBP, which presumably boosts p53 transcriptional activity (34). The argument against this canonical model starts from the work done by Vousden and Blattner in 1999 (4, 8). They both proposed that p53 can be stabilized regardless of its phosphorylation status (4, 8). The more recently described Ser18Ala and Ser23Ala knock in mice question even more the significance of these modifications in the regulation of p53 stability. Ser18Ala or Ser23Ala knock in mice exhibited only mild defects in p53 stability regulation (16, 135, 150), and even the double mutant mouse showed very limited defects in certain tissues, with no major physiological changes in mouse embryonic fibroblasts and most adult tissues (17). More and more evidence supports the idea that p53 phosphorylation is dispensable for its protein stability. Instead, the growing evidence supports that p53 phosphorylation does impact its transcription activity. A more conservative interpretation might be that p53 phosphorylation only plays a major role in the regulation of p53 in certain tissues or under specific circumstances.

1.4.3. Acetylation

The covalent linkage of an acetyl group on lysine residues was first observed on histones. Acetylation neutralizes the positive charge on histone proteins, and histone acetylation is believed to allow greater access of the general transcriptional machinery to condensed chromatin. p53 was the first reported acetylated non-histone protein (56). P53 is acetylated by the histone acetyltransferase CBP/p300 at six lysine

sites located in the COOH –terminus, and acetylated p53 exhibits dramatically higher sequence specific DNA binding activity(103). The function of COOH-terminal p53 acetylation was tested at the organism level with generation of the p53 “6KR” knock in mouse, where all 6 COOH-terminal acetylation sites are mutated to arginine. The 6KR mouse demonstrates impaired gene expression only at selected p53 transcription targets and in certain tissues (43).

Recent, more sophisticated p53 acetylation analysis utilizing mass spectrometry revealed that two additional p53 lysines could be acetylated. p53 K120 is acetylated by hMOF and Tip60 based on both in vitro and in vivo data(139, 140). Unlike other p53 lysines, K120 is highly conserved among all species carrying a functional p53 gene. p53 acetylated at K120 selectively activated the pro-apoptotic gene targets PUMA and BAX, but not MDM2 and p21 [39]. Like K120, another new acetylation site, K164, is also located in the p53 DNA binding domain (141). K164 acetylation is presumably critical for the tumor repressor function of p53 since an acetylation defective mutation at this site has been found in human tumor samples. Simultaneous loss of acetylation on K120, K164 and the C-terminal six lysines completely abolished p53 transcriptional activity on a wide range of p53 responding promoters (141). Thus, p53 acetylation, in contrast to phosphorylation, is required for its transcriptional activation activities.

In addition to the eight acetylated lysines noted above which have been identified in

vivo and linked to p53-mediated transcriptional activation, K320 acetylation was also identified, but as an in vitro target of P/CAF (p300/CBP association factor) (101). To test the function of this acetylation, a K317R knock-in mouse was generated. Surprisingly, increased expression of p53 pro-apoptotic genes was observed in this mouse (18). Taken together, the accumulated data on the function of p53 acetylation reveals that there are a diversity of functions among p53 acetylation sites, and much remains to be clarified in this area of p53 research.

1.5 p53, cell cycle arrest and apoptosis

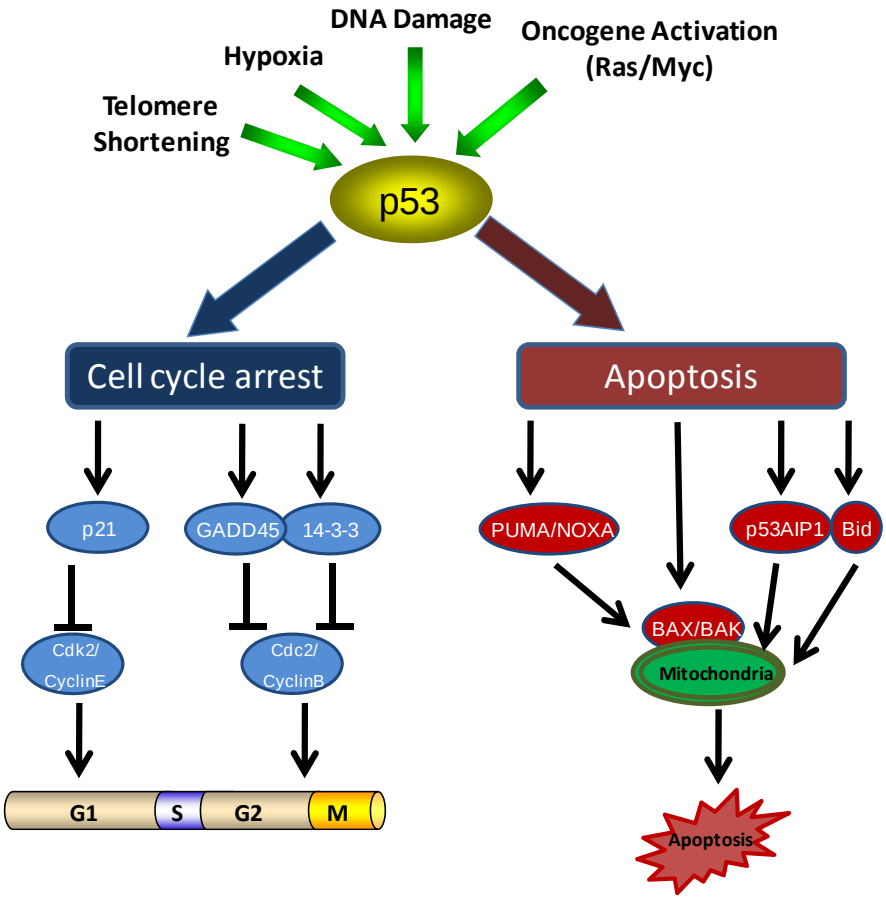
Cells are frequently exposed to environmental and intrinsic stress factors. When mild injury happens, cell growth pauses to allow time to repair the damage. Extensive damage, however, will initiate a cell death program in the heavily damaged cells to prevent potential oncogenic jeopardy. For many cell stressors, p53 will control the life or death decision process. p53 can choose to coordinately activate cell cycle arrest and DNA repair pathways after limited damage or choose to remove heavily damaged cells via the apoptosis pathway. How p53 "knows" which genes to turn on or off to benefit the organism is a topic of intense interest.

Kastan and colleagues first noted that ATM, p53, and GADD45 compose a signal transduction pathway to control the mitosis checkpoint upon DNA damage (77, 87). The dysfunction of this pathway is also coupled with tumor progression (77, 87). Later, p53 was also linked to G1 arrest in response to DNA damage due to its induction of

p21, a cyclin-dependent kinase inhibitor (37). MEFs derived from p21 knockout mice have a near complete defect in G1 phase arrest after DNA damage (29). p53-dependent apoptosis involves the activation of many more genes than G1 arrest, including PUMA, NOXA, BAX, BAK, Bid, P53AIP1, Apaf, PERP, among others. PUMA has emerged out as the most critical mediator of p53 apoptotic function, since PUMA knockout mice demonstrate the same defect in stress-induced apoptosis that has been observed in p53 knockout mice (71). Even though p53 can mediate apoptosis in a transcription independent manner(145), its transcriptional activity plays the dominant role in this stress response. A simplified graphic description of the p53 signaling network regulating cell cycle arrest and apoptosis is shown in Fig.1.1.

Fig. 1.1 Participation of p53 in cell cycle arrest and apoptosis.

p53 induces p21, resulting cell in cycle cycle arrest in G1 phase. p53 also contributes to G2/M arrest through the activation of GADD45 and 14-3-3. p53 induced apoptosis due to the transcription-dependent mechanism is shown in a simplified manner with only downstream, target genes of the mitochondrial apoptosis pathway (PUMA, NOXA, BAX, BAK etc.) displayed.



How does p53 decide to promote apoptosis versus cell cycle arrest? How does p53 tip the balance between life and death? Often, the decision appears to be determined by the “epigenetic code” of p53 covalent modifications or the presence or absence of p53 cooperating factors. A few selected examples will be mentioned to represent the current models.

1.5.1 Covalent modifications

The N-terminal transactivation domain of p53 is highly regulated by phosphorylation. Ser 46, one out of the eleven p53 phosphorylation sites, has a distinctive function in regulating the arrest/death decision after DNA damage. Ser46 phosphorylated p53 specifically activates pro-apoptotic genes such as PIG3, BAX and p53AIP1, but not cell cycle arrest genes (63). This finding is strongly supported by the phenotype of S46A knock in mouse. p53-dependent apoptosis is partially impaired in thymocytes and mouse embryonic fibroblasts (MEFs) of S46A knock in mouse after DNA damage. Consistent with this phenotype, transcription of p53 target apoptotic genes is preferentially affected by S46A mutation after DNA damage in MEFs of this mouse (42). Ser46 is a target for multiple kinases, including HIPK2(24), DYRK2(122) and AMPK(116). The multiple kinases might phosphorylate p53 at Ser46 under various DNA damage or other stress conditions. The mechanism by which Ser46 phosphorylated p53 favors apoptotic gene activation, however, remains unknown.

Acetylation also regulates the promoter preference of p53. K120 acetylation of p53 leads to preferential induction of apoptotic genes over growth arrest genes (140) with the mechanism unknown. K320 modification also regulates the apoptosis/arrest decision by p53, but via both acetylation and ubiquitination, and conflicting data can be found in the literature. Le Cam has reported that antagonism between ubiquitination and acetylation of this site is what determines cell fate after stress (92). E4F1, an atypical E3 ubiquitin ligase catalyzing a non-degraded K48-linked ubiquitin chain on p53 K320, competes with P/CAF, the acetylase targeting K320 (92). E4F1 overexpression with resulting high level K320 ubiquitination induces cell cycle arrest via increased activation of p21. In a separate study, acetylation on K320 caused the preferential activation of genes containing high-affinity p53 binding sites, mainly cell cycle arrest genes (83). Furthermore, The K317R (Human K320R) knock-in mouse model suggests K320 acetylation represses p53 apoptotic gene expression (18). Finally, K373 acetylation, one out of the six acetylation sites in the p53 C-terminal fragment, promotes activation of the apoptotic gene program, though not at the expense of cell cycle arrest gene expression, such as p21 (83). Thus, the functional sequence of the acetylation pattern on p53 might be compared with the modification-based “histone code” that specifies the transcriptional activity of chromatin regions, as each unique pattern of acetylation influences a specific biological activity of p53.

1.5.2 Co-factors

Besides the covalent modifications on p53 itself, p53 interacting partners appear to be implicated in the modulation of p53 transcriptional activity. The co-factor proteins either bind to sites adjacent to p53 response elements to induce specific gene expression, or interact with p53 protein itself to influence the gene target preference of p53. The transcription cofactor JMY, in collaboration with p300, facilitates the selective activation of BAX, but not p21 (132). ASPP family proteins(ASPP1 and ASPP2) have been reported to augment p53 binding activity such that p53 preferably activates proapoptotic genes, which normally contain low affinity p53 binding sites in their promoters (126). The inhibition of ASPP proteins indeed blocks the expression of the apoptotic p53 target genes BAX and PIG3 (133). In contrast, Hzf, a hematopoietic zinc finger protein, directly interacts with the p53 DNA binding domain, and promotes the recruitment of p53 to the p21 and 14-3-3σ promoters to block cell cycle progression (25). Hzf also simultaneously attenuates the gene expression of PUMA and BAX [60] to favor the growth arrest cell fate.

In summary, both covalent modifications and co-factors are involved in helping p53 make the life-or-death decision. The prediction of p53 function relies on p53 modification, absence or presence of p53 co-factors, cell type and cellular microenvironment. The better understanding of each specific situation will aid us in creating different therapeutic strategies to kill cancer cells via p53 manipulation.

1.6 p53 has direct pro-apoptotic functions at the mitochondria.

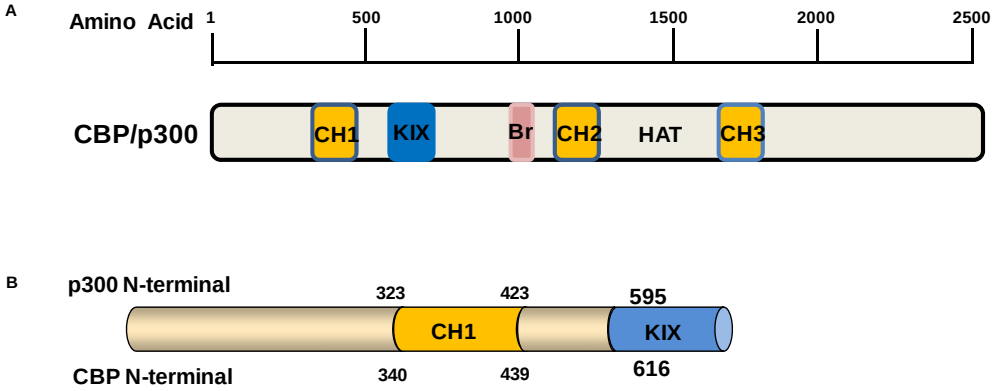
p53 induced apoptosis is dependent on the expression of its pro-apoptotic genes. However, the transcriptionally inactive mutant p53 (22/23QS) acts as a powerful death inducer in tumor cells(85). Recent work by Moll and colleagues has characterized the mechanism of transcription-independent apoptosis as being due to direct action by p53 on the mitochondria. A distinct pool of cytoplasmic mono-ubiquitinated (mono-ub) p53 translocates to mitochondria after a stress stimulus. p53 then undergoes deubiquitination mediated by the deubiquitinase HAUSP that generates apoptotically active non-ubiquitinated p53. The active mitochondrial p53 increases the permeability of the outer mitochondria membrane by forming a complex with membrane protective proteins (Bcl-2/ BclXL), which induces cytochrome C release to trigger apoptosis (105, 109). The physiological significance of this pathway is reflected by the mutation hot spots in p53 in breast cancer. p53 with mutation on H175, H273 or K 280, which is deficient in DNA binding, retains the ability of mitochondria translocation but loses the interaction with Bcl2/BclXL proteins (109). That the selective p53 mutations in tumors not only silence the p53 transcription activity, but also abrogate the p53 transcription-independent apoptosis is the compelling evidence supporting the physiological significance of this novel p53 apoptotic pathway.

1.7 CBP/p300, and p53

CBP and p300 are chromatin remodeling proteins. They enhance transcription activities of 10% of the transcription factors of the human genome through three conceivable mechanisms. First, these ~2500 amino acid long proteins act as scaffolds to bridge transcription factors and the RNA pol II holoenzyme (51). Second, CBP and p300 possess an intrinsic histone acetyltransferase activity that acetylates histone tails, leading to increased access of the transcriptional machinery to genes requiring activation in condensed chromatin(76). Third, CBP and p300 can extend their acetylase activity to transcription factors themselves and increase their specific DNA binding ability(56). p53 was the first identified non-histone protein substrate of CBP/p300 HAT activity(56). p53 acetylation not only competes for ubiquitination to stabilize p53 but also increases the DNA binding affinity of p53. CBP and p300 are therefore defined as transcriptional coactivators of p53.

Fig. 1.2 The domain structure of CBP and p300 protein.

(A) The functional domains in CBP and p300 are indicated, including the cysteine/histidine-rich domains CH1, CH2 and CH3, the KIX domain, the bromodomain (Br) and histone transferase domain (HAT). (B) The domains in CBP and p300 N-terminus. The size of each protein or protein fragment is indicated in number of amino acid residues.



p300 may also have a distinctive function in facilitating p53 degradation. p300 interacts with both MDM2 and p53 independently and they together form a tripartite complex (55). An MDM2 mutant deficient in p300 binding failed to degrade p53 (55). Furthermore, recombinant p300 protein was characterized as an E4, with the ability to conjugate poly-ubiquitin chains, a traditional proteasome degradation signal, onto the mono-ubiquitin modified p53 (54). Hence p300 is predicted to operate in concert with MDM2 to influence normal p53 turnover. However, prior studies did not study p300 E4 or degradation functions for p53 under physiological conditions. Also, the p300 paralog CBP was not characterized for these functions either. How does p300 or CBP, reconcile two seemingly opposite regulatory functions, ubiquitination activity versus acetylation activity? Does one function override the other under different environmental conditions?

1.8 The regulation of p53 degradation by the ubiquitin-proteasome system.

The proteasome degradation of p53 is mostly ubiquitin dependent although a ubiquitin independent mechanism has been reported (5). A multitude of E3 ubiquitin ligases of p53, including MDM2, E4F1, COP1 and Arf-BP1, have been mentioned in section 1.5. It is unclear how each E3 contributes to overall p53 regulation in any given cell-type or environmental condition. Adding to difficulty of understanding p53 ubiquitination-proteasome system, the additional layers of regulation are proposed

beyond the E3s.

1.8.1. E4 ubiquitin ligase

Multiubiquitin chain assembly requires the collaboration of E1 activation enzyme, E2 conjugation enzyme and E3 ubiquitin ligase. Recently, a new enzyme activity of specific ubiquitin-chain elongation or E4 activity was described (65). E4 ubiquitin ligase targets monoubiquitinated (mono-Ub) or oligoubiquitinated(oligo-Ub) proteins, which are modified with single ubiquitin on one or multiple lysines, as the enzymatic substrates. E4 proteins bind to monoubiquitinated (mono-Ub) or oligoubiquitinated(oligo-Ub) substrates and catalyze multiubiquitin chain assembly along with E1, E2 and in some cases, a collaborating E3 (65). The first identified E4 ligase, yeast UfD2, contains a U-box (UFD2-homology domain) domain which shares structural similarity with the canonical E3 RING finger domain (84). Besides UfD2, CHIP (C terminus of Hsc70-interacting protein) is another U-box-containing protein that can function as an E4 with Parkin, a RING finger E3 ligase (68). p300 and BUL1/BUL2 complex have been reported as possessing E4 ligase activity but do not contain a U-box or any sequence related to the catalytic domain of known ubiquitin ligases (54, 65). It is well accepted that mono-ubiquitin and oligo-ubiquitin conjugates have non-proteolytic function, instead regulating protein translocation or transcriptional activity (58). Thus, E4 ubiquitin ligases might be important not only for promoting protein degradation, but also for modulating signal transduction dependent

on specific ubiquitin chain configurations.

Monoubiquitinated p53 physiologically exists and has distinctive functions distinct from native or poly-ubiquitinated p53. Once p53 is tagged with one ubiquitin, the nuclear export signal (NES) becomes exposed, leading to the translocation of p53 from nucleus to cytoplasm (95). Upon stress, monoubiquitination of p53 could serve as a mitochondrial relocation signal perhaps due to conformational change. The distinct cytoplasmic pool of stable monoubiquitinated p53 could face two fates, inactivation via subsequent polyubiquitination and degradation in unstressed cells, or activation as a death trigger via mitochondrial targeting upon stress. p53 E4 ligases, if they exist, may very well control this binary fate of cytoplasmic monoubiquitinated p53.

Another aspect of E4 function critical to regulation of their targets is the identity of the Ub chain linkage in the polyubiquitin chains catalyzed by the E4 activity. Multiubiquitin chain assembly uses many different Ub lysines for the ubiquitin-ubiquitin linkages. K48 linked chains are the most common type, this linkage is the proteasome degradation signal. K63 linked chains are also common, but are involved generally in signal transduction or cellular trafficking. K63 chains have been observed on p53 protein and cause its sequestration in the cytoplasm (89). A p53 E4 might serve as the determining factor of the ubiquitin chain type for p53 based on cellular localization or environmental factors.

1.8.2. The delivery of p53 to the 26S proteasome

The 26 S proteasome is a multi-subunit complex composed of a barrel like 20S catalytic core (CP), whose proteolytic activity is embedded in the inner wall of the chamber to prevent self-degradation, and two regulatory 19S regulatory particles (RP) which are responsible for recognizing, unfolding, and translocating polyubiquitinated substrates into the 20 S proteasome for degradation(48). The 19S RP has nine subunits comprising the "lid" and ten subunits comprising the "base". The six ATPase subunits (RPT1-RPT6) in the base, which dock directly onto the 20S, unfold protein substrates and control the open-closed state of the proteasome channel to transport protein substrate to the proteolytic milieu of the 20S for degradation(114). The other four non-ATPase subunits in the base are Rpn1, Rpn2, Rpn10 and Rpn13. Critical to the function of the proteasome is the recognition of the ubiquitin chains of protein substrates. Rpn1 interacts with a family of ubiquitin chain receptor proteins, all of which share a conserved UbL (ubiquitin like) domain and one or two UBA (ubiquitin association) domains (40). The UBL domain interacts with the proteasome and the UBA domains specifically bind to K-48 linked polyubiquitin chains. These proteins, Rad23, Dsk2 and Ddi1, are proposed to work as shuttles to deliver polyubiquitin tagged substrates to Rpn1. Surprisingly, Rpn1 is not essential for viability in yeast (114).

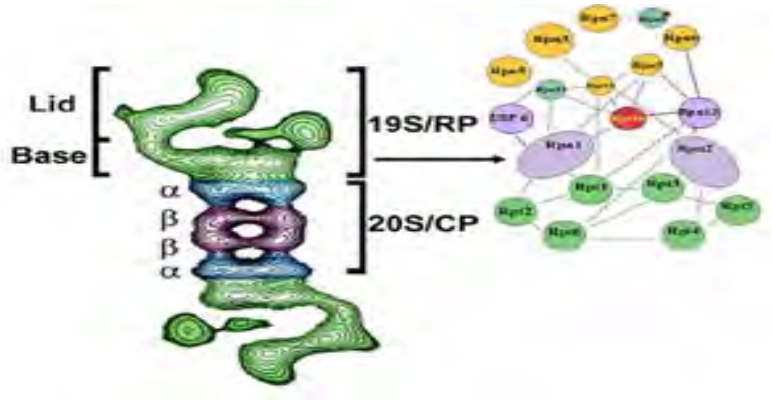
Rpn10 (mammalian S5a), carrying a UIM (Ubiquitin Interacting Motif) domain in its N-terminal end, is another ubiquitin-binding proteasome subunit (31). Rpn10/S5a

interacts with Rad23 proteasome adaptor proteins (62, 104). Like Rpn1, Rpn10 knockout yeast strains have almost no phenotype(147), raising a question as to the physiologic importance of this subunit for substrate recognition by the proteasome. Rpn13 is yet another Ub (K48 chain) binding subunit of the 19S RP, but it, too, is not essential for the yeast viability (67). Although ubiquitin and the proteasome are absolutely vital for cell viability in yeast or mammalian cells, the knockout yeast strains of these potential ubiquitin recognizing factors are not lethal. Only functional redundancy or additional ubiquitin-interacting subunits could explain the discrepancy. These findings are all based on work in yeast, and much remains to be done to explore the function of these proteasome subunits in mammalian cells to see if their functions are comparable across the evolutionary spectrum.

Thus, the mechanism of protein substrate delivery into the proteasome remains relatively unknown. From what is understood from the literature , the ubiquitin interacting proteasome subunits (Rpn1, Rpn10/S5a, Rpn13) might work alone, or cooperate with the proteasome shuttling proteins (Rad23, Dsk2, Ddi1) to deliver protein substrates to the 26S proteasome. The increasing number of proteins in the proteasome delivery network indicates that this post-ubiquitination step might provide substrate selectivity to proteasome delivery, as has already been demonstrated recently for a few substrates in yeast [83].

Fig. 1.3 The 26S proteasome (Adapted from (114))

(Left) 2D model of the 26S proteasome generated through averaging of electron micrographs. (Right) Schematic representation of the 19S RP. The 26S proteasome is composed of one 20S CP (lower) and two 19S RP particles. The regulatory particle can be further sub-divided into the 'lid' and the 'base'. The subunit organization of the 19S particle was adapted from (46).



Both hHR23A/B (human Rad23) and hPlic (human Dsk2), proteasome adaptor UbL-UBA proteins, have been reported to regulate p53 turnover (10, 81), probably due to their ability to interact with both the ubiquitinated p53 and the proteasome. It would be interesting and clinically valuable to figure out the docking site subunit on the 26S proteasome in the p53 delivery pathway. Considering that reactivating p53 is a commonly used idea in anti-cancer drug design, the finding of the proteasome subunit critical for p53 degradation will provide a novel cancer drug target.

1.9 Activating p53 for tumor therapy

p53 is frequently mutated in human cancers and its mutation happens in more that 50% of human tumors. Tumors bearing wild type p53 are often coupled with MDM2 and/or MDM-X amplification, or the loss of ARF (148). Reactivating p53 in these tumors could be used as a therapeutic approach. This approach is supported by an animal model study demonstrating that restoration of wild type p53 leads to efficient tumor regression(151). p53 activation could theoretically be achieved by the inhibition of the general proteasome degradation system, or through the disruption of the p53-specific ubiquitination and degradation pathway.

1.9.1. Targeting the general proteasome degradation system.

Proteasome inhibitors can be used as cancer drugs. For example, the proteasome inhibitor Velcade has been approved for treatment of multiple myeloma (114). Multiple signal transduction pathways are involved in the anti-tumor activity of

proteasome inhibitors. The major survival gene NF-κB can be inhibited due to the blocked degradation of IκB-α, a repressor of NF-κB (1). In some circumstances, the abrogated p53 degradation and the subsequent augmented p53 protein level as well as the increased p53 transcription activity explain the induced apoptosis when cancer cells were treated with proteasome inhibitors(1). The first FDA approved proteasome inhibitor drug, Bortezomib, is now used to treat myeloma and is under the evaluation for treating other solid tumors(1).

UBE1, the ubiquitin activating enzyme was the only known E1 in mammals until the second E1, UBA6 (ubiquitin-like modifier activating enzyme), was recently discovered (73). Targeting E1 will lead to general reduction in protein degradation so the inhibitor of E1 is worth investigating for anti-tumor drug development. A small chemical inhibitor of E1, PYR-41, has been synthesized in Alan Weissman's group and has been reported to be capable of killing transformed cells bearing wild-type p53 (154). The molecular basis of PYR-41 induced cell death includes both the inhibited NF-κB activity and the augmented activity of p53.

More than forty human E2 conjugating enzymes have been discovered to date. Inhibiting E2 should provide increased the specificity for the affected protein substrates compared to inactivating E1 or proteasome. A very interesting discovery about Ubc13, the K63 chain specific E2 conjugating enzyme, demonstrated that the association of p53 with Ubc13 results in increased p53 ubiquitination and the reduced

p53 tetramerization, thereby inhibiting p53 transcriptional activity(143). This work suggests that the inhibition of Ubc13 should cause the increased p53 transactivity. Small molecule inhibitors of Ubc13 are under the investigation for anti-tumor drugs.

1.9.2 Targeting the multiple steps of p53 ubiquitination and the proteasome degradation pathway.

Targeting p53 E3 has a huge therapeutic advantage in terms of the specificity to achieve p53 activation. Generally, E3 ubiquitin ligases are the most common cancer genes next to protein kinases, and frequently are oncogenes. In certain tumors, MDM2 is amplified to repress p53 tumor suppressor function. Blocking MDM2 in certain situations is like killing two birds with one stone. Several different strategies have been used to design MDM2 inhibitors: 1) Detaching MDM2 from p53 by interfering with their interaction; 2) Targeting MDM2 intrinsic ligase activity;3) Reducing MDM2 expression(1). The chemical inhibitor Nutlin3a, which binds to the p53 binding pocket on the MDM2 N-terminus, is able to disrupt the p53-MDM2 interaction (22). Nutlin3a primarily induces cell cycle arrest thereby exhibiting anti-tumor activity. Pioneered by the work of MDM2 inhibitors, the methodology of targeting other E3s of p53, or even the potential E4 (p300) of p53, is predicted to be effective in anti-tumor activity.

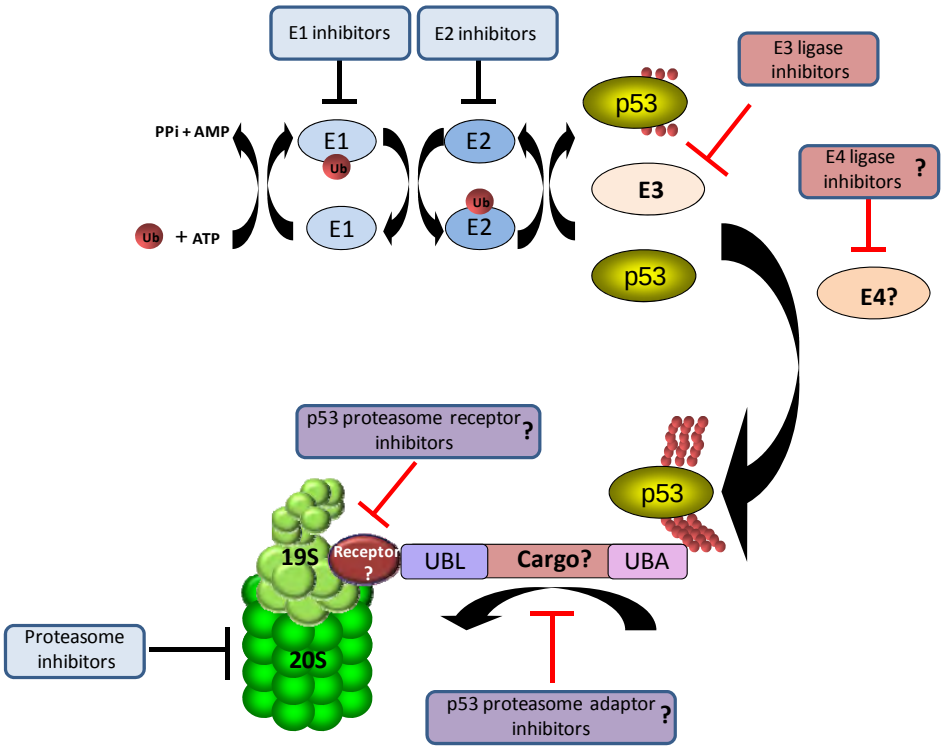
Beyond E3, proteasome adaptor UBL-UBA proteins, Rad23 and Dsk2, are presumed to be the carriers of polyubiquitinated p53. The growing number of UBL-

UBA proteins indicates this protein family might have some substrate specificity, even not the stringent one to one specificity, but the relatively modest specificity of targeting a subset of protein substrates (60, 96). Screening for inhibitors of Rad23 or Dsk2 might be a novel approach to find p53 activators to kill tumor cells.

In addition, the existence of multiple ubiquitin-interacting proteasome subunits suggests that there might be different entry gateways on the proteasome for the poly ubiquitinated protein substrates or the UBL-containing cargo proteins. Targeting the entry point of p53 on the proteasome is not only advantageous with respect to drug potency since the proteasome receptor of p53 initiates the signal cascade downstream to p53 degradation, but is also might be less toxic when compared with other general 20S inhibitors of the proteasome.

Fig.1.4 The strategy of activating p53 by inhibiting ubiquitination and proteasome degradation of p53.

The color of the rectangular shape indicates the specificity of the inhibitors in p53 activation. Blue: generic inhibitor; red: specific inhibitor; purple: relatively specific inhibitors.



1.10 Concluding Remarks

Thirty years of p53 research has generated over 50000 research articles, which provide extraordinarily abundant information. But the plentiful knowledge of p53 does not necessarily clarify our understanding of the regulation of p53 by the ubiquitin/proteasome system. Even to a simple question, the answers are often strikingly different in different model systems. p53 is not a simple switch to control different cell fates. It is a central node in a very intricate signaling network, receiving various input signals and controlling diverse cellular functions with the interaction of many other players. The final output of this network is not only determined by p53 itself, but also by other modulators of p53, for example, the molecules catalyzing p53 modifications, other transcription cofactors of p53, etc.

p300/CBP act as multifunctional regulators of p53 via acetylase and ubiquitin ligase activities. The acetylase activity positively regulates p53 transactivation and the ubiquitin ligase activity, though only supported by in vitro evidence, might negatively regulate p53. Is the ubiquitin ligase activity of p300 (and CBP?) a physiological activity? How does p300 reconcile two seemingly opposite regulatory functions; ubiquitination activity and acetylation activity? Does one function override the other in different situations? And, once ubiquitinated, how exactly is p53 delivered to the proteasome? The experiments in this thesis were designed to seek the answers to these questions.

Chapter 2: CBP and p300 are physiological E4 ubiquitin ligases of p53

2.1 Abstract

p300 and CREB-binding protein (CBP) act as multifunctional regulators of p53 via acetylase and polyubiquitin ligase (E4) activities. Prior work in vitro has shown that the N-terminal 595 aa of p300 encode both generic ubiquitin ligase (E3) and p53-directed E4 functions. Analysis of p300 or CBP-deficient cells revealed that both coactivators were required for endogenous p53 polyubiquitination and the normally rapid turnover of p53 in unstressed cells. Unexpectedly, p300/CBP ubiquitin ligase activities were absent in nuclear extracts and exclusively cytoplasmic. Consistent with the cytoplasmic localization of its E3/E4 activity, CBP deficiency specifically stabilized cytoplasmic, but not nuclear p53. The N-terminal 616 aa of CBP, which includes the conserved Zn^{2+}-binding C/H1-TAZ1 domain, was the minimal domain sufficient to destabilize p53 in vivo, and it included within an intrinsic E3 autoubiquitination activity and, in a two-step E4 assay, exhibited robust E4 activity for p53. Cytoplasmic compartmentalization of p300/CBP's ubiquitination function reconciles seemingly opposed functions and explains how a futile cycle is avoided—cytoplasmic p300/CBP E4 activities ubiquitinate and destabilize p53, while physically separate nuclear p300/CBP activities, such as p53 acetylation, activate p53.

2.2 Introduction

p53, or a component of its tumor suppressor pathway, is mutated or dysregulated in nearly all human cancers (108). Depending on context, it can signal cells to arrest, senesce, or apoptose through both transcription-dependent as well as independent mechanisms (110, 134). Its activity is controlled posttranslationally by a multitude of covalent modifications, including phosphorylation, ubiquitination, sumoylation, neddylation, methylation, and acetylation (9, 111).

The ubiquitination and proteasome targeting of p53 by the RING E3 enzyme MDM2 has been considered to generally inhibit p53 function (12). However, the distinction between multiple monoubiquitination (MUM) and polyubiquitination of p53 adds additional complexity to its regulation. MUM has been reported to enhance p53 nuclear export, and in the absence of stress, p53 is very likely polyubiquitinated and then degraded by the proteasome in the cytoplasm (13, 95). In addition, recent evidence points to a potential positive role for p53 ubiquitination in its capacity as a nuclear transcription factor (9, 78, 92). Adding to the difficulty of understanding p53 ubiquitination, a multitude of ubiquitin ligases (E3s) have been identified for p53 in unstressed cells besides MDM2, most notably E4F1 (33, 92), COP1 (33), and ARF-BP1 (20), making it unclear how each E3 contributes to overall p53 regulation in any given cell-type or environmental condition (11).

Whether p53 MUM vs. polyubiquitination is actively and specifically controlled

in cells also remains unclear. In stressed cells, the induction of MDM2 expression by activated p53, can specifically increase the abundance of polyubiquitinated p53 adducts (95). In unstressed cells, specific polyubiquitin ligases (E4s), or ubiquitin (Ub) chain-extending factors, exist for p53—namely the coactivator p300 (54) and transcription factor YY1 (138). p300-dependent polyubiquitination of p53 in vitro required priming of p53 by MDM2-driven p53 MUM (88), although p300, and its paralog CREB-binding protein (CBP), both exhibited robust E3 autoubiquitination activity (54). YY1, by contrast, stabilized MDM2/p53 complexes, allowing MDM2 to act more processively toward chain elongation (138). A recognizable E3 or E4 domain could not be identified in p300/CBP, but the N-terminal 595 aa of p300 appeared to harbor both its E3 (autoubiquitination) and E4 activities (54). Recently, Zn-finger like domains have also been characterized as active E3 enzymes (92, 149), and p300 does harbor three conserved Zn^{2+}-binding Cys-His-rich regions, one of which (C/H1-TAZ1) lies within its first 595 aa (3, 26, 49).

To better define the role of p300 or CBP in p53 stability regulation, p53 turnover was studied in p300- or CBP-deficient cells. p300 or CBP depletion in unstressed cells led to p53 stabilization, and p300 and CBP E3 activities were exclusively cytoplasmic, with detectable amounts of both proteins localized to the cytoplasm. CBP, like p300, encoded an E3 activity within its N terminus and exhibited E4 activity toward p53 in vitro. p300 and CBP therefore engage in compartmentalized regulation of p53, by cytoplasmic ubiquitination and presumed nuclear acetylation, that is required for the

proper homeostasis of p53 in basal and stressed conditions.

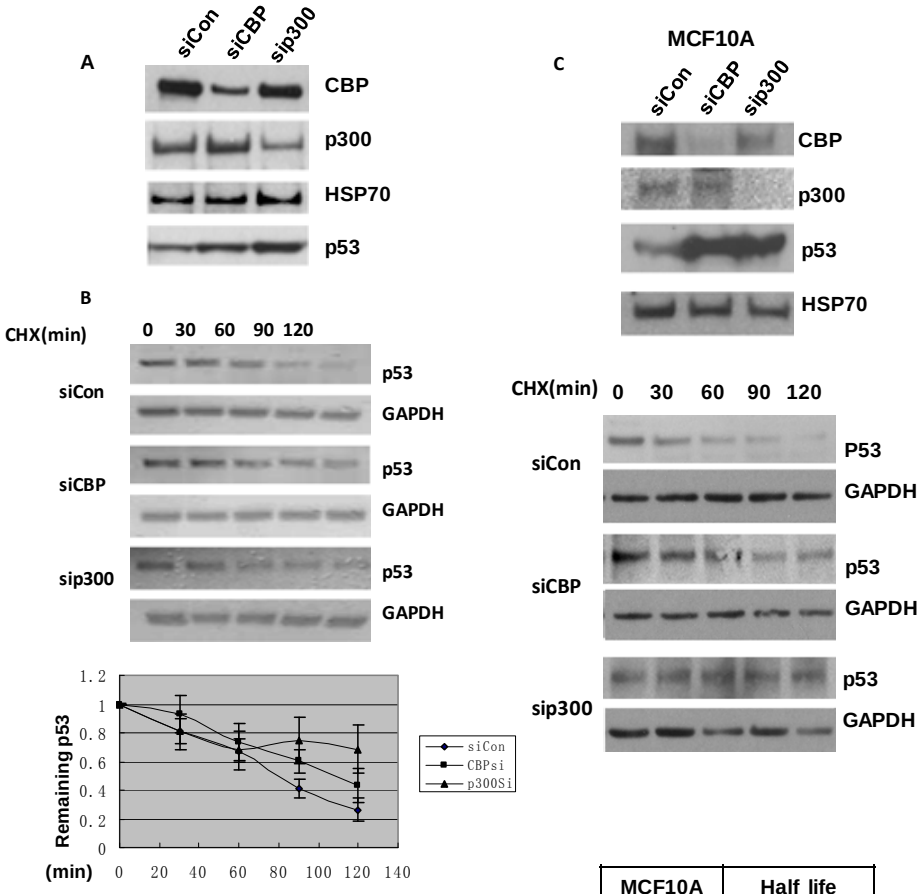
2.3 Results

Regulation of p53 Abundance and Stability by p300 and CBP.

To assess the physiologic contributions of p300 and CBP to p53 regulation in unstressed cells, p300 and CBP were transiently silenced in U2OS cells with specific siRNA duplexes, followed by analysis of cell lysates for p300, CBP, and p53 levels, as well as determination of p53 half-life (t1/2) by cycloheximide decay (Fig. 2.1 A and B). p300 siRNA has been reported to increase p53 abundance but the mechanism was not determined further (98). p300 and CBP siRNAs both caused increases in steady-state p53 abundance (Fig. 2.1. A) and p53 half-life (from 75 min to >2 h and 2 h, respectively; Fig. 1B) compared to that seen in control siRNA-treated cells. Demonstrating that these results were not cell type-specific or due to the use of a cancer cell line, siRNA depletion of CBP or p300 in nontransformed MCF10A human breast epithelial cells similarly stabilized p53 from t1/2 = 75 min with control siRNA to >2 h for both p300 and CBP siRNAs (Fig. 2.1. C).

Fig. 2.1 CBP and p300 regulate p53 stability.

(A and B) p53 abundance and stability in U2OS cells after CBP or p300 siRNA treatment. (A) U2OS cells transiently transfected with the indicated siRNAs for 72 h were harvested for immunoblotting with anti-CBP, -p300, -hsp70 (loading control), and -p53 antibodies. (B) (Top) U2OS cells transfected with the indicated siRNAs for 72 h were treated with cycloheximide, and lysates were harvested at the indicated times for analysis by p53 immunoblot. (Bottom) p53 levels were quantitated on a blot scanner (LiCOR), and half-life calculated based on the decay of normalized (to GAPDH loading control) p53 levels to 50% of their original level. Values are an average of three independent experiments. Error bars, ±1 standard deviation (S.D.). (*C*) Effect of CBP or p300 siRNA on p53 stability in MCF10A breast epithelial cells. MCF10A cells transiently transfected with the indicated siRNAs for 72 h were treated with cycloheximide and lysates harvested at the indicated times. (Top) The lysates from the time 0 min of the cycloheximide treatment were blotted with CBP, p300, p53, and HSP70 antibodies. (Middle) Lysates from the cycloheximide time course were analyzed by p53 immunoblot. (Bottom) p53 half-life was calculated as in Fig. 1B.



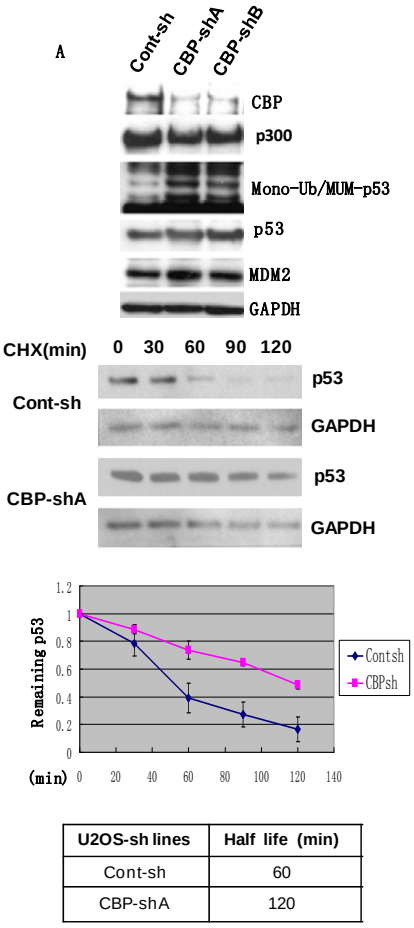
U2OS siRNA	Half life (min)
siCon	75
siCBP	120
sip300	>120

MCF10A SiRNA	Half life (min)
siCon	75
siCBP	>120
sip300	>120

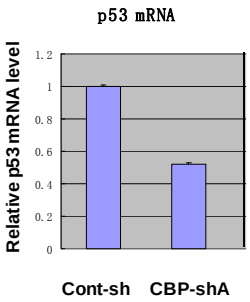
Indirect explanations for the increased p53 abundance and stability induced by p300/CBP depletion, such as increased p53 mRNA expression, decreased MDM2 expression, or induction of genotoxic stress (the latter two of which might stabilize p53) were investigated. RT-PCR analysis showed that p53 mRNA levels were actually decreased, not increased, in CBP-shA as compared with control-sh cells (Fig. 2.2. B), and MDM2 protein levels were increased or unchanged, not decreased, in CBP-shA or CBP-shB cells as compared with control-sh cells (Fig. 2.2 A). The possibility that CBP depletion might stimulate a p53-stabilizing stress response (118) was investigated by mock or doxorubicin (Dox)-treatment (as positive control for stress stimulus) of control-sh or CBP-shA cells, followed by immunoblotting of lysates for p53 serine 15 (S15) phosphorylation, a marker of p53 activation in response to stress (Fig. 2.2 C) (80, 131). The untreated cell lines had an equivalent and very low level of serine15 phosphorylation, and both showed robust induction of phosphorylation after treatment with Dox for 2 h (Fig. 2.2 C). Thus, the lack of basally induced p53 S15 phosphorylation in unstressed CBP-depleted cells does not support a nonspecific stress mechanism as the reason for the observed p53 stabilization.

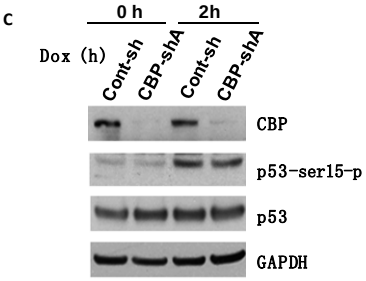
Fig. 2.2 CBP regulates p53 degradation at the post-translational level.

(A) (Top) p53 abundance in CBP stably deficient U2OS cells. Lysates of drug-selected clones of U2OS cells harboring the indicated control (empty vector) or CBP shRNAs were analyzed by immunoblotting with anti-CBP, anti-p53, anti-MDM2, and anti-GAPDH antibodies. (Bottom three panels) The indicated shRNA-expressing cell lines were treated with cycloheximide and lysates harvested at the indicated times and analyzed by p53 immunoblot. p53 levels were quantitated by densitometry, and half-life calculated based on decay of normalized (to GAPDH loading control) p53 levels to 50% of their original level. Values are an average of three independent experiments. Error bars, +/- 1 S.D. (B) p53 mRNA level in control and CBP shRNA expressed U2OS cells. p53 mRNA abundance in control and CBP-shA U2OS cells was assessed by QRT-PCR. Each sample was internally standardized to GAPDH. Relative expression levels (with standard errors of the mean) are presented. (C) p53-ser 15 phosphorylation in CBP-shA cells. Mock or Dox (2 M) treated control-sh or CBP-shA cells were immunoblotted for p53 phosphoserine 15, total p53, CBP, and HSP70 (loading control) levels.



U2OS-sh lines	Half life (min)
Cont-sh	60
CBP-shA	120





CBP and p300 are required for p53 polyubiquitination.

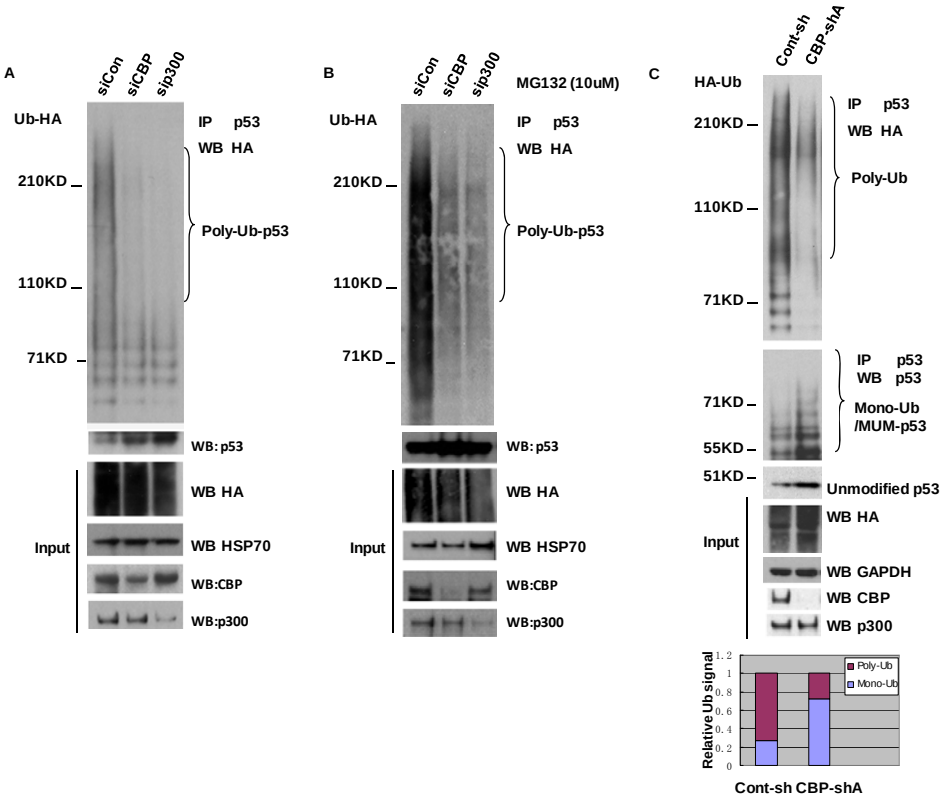
Given that p300 can act as an E4 for p53 in vitro (54), the abundance of p53-Ub conjugates was analyzed in U2OS cells expressing HA-tagged Ub after control, CBP, or p300 siRNA treatment, and in the presence or absence of MG132 proteasome inhibitor. Lysates of siRNA-treated cells were analyzed by p53 IP under stringent conditions, followed by HA immunoblot (Fig. 2.3 A and B). Polyubiquitinated p53-Ub conjugates were detected in a p53 IP of lysate from control siRNA-treated cells, as evidenced by the broadly distributed HA immunoreactive species (Fig. 2.3 A, first lane). Based on the pattern of p53-Ub conjugates seen in vitro when non-chain-forming Ub moieties are used, those p53-Ub conjugates <100 kDa represent largely mono-Ub and MUM species, while those >100 kDa represent polyubiquitinated species (54). p53 IPs from lysates of p300 or CBP siRNA-treated cells, by contrast, had significantly reduced levels of polyubiquitinated p53 (Fig. 2.3 A, second and third lanes). Similar results were seen after proteasome inhibition, except for a stronger polyubiquitinated signal in the p53 IP from control siRNA-treated cells (Fig. 2.3 B). Similar results were also seen when the p53 ubiquitination pattern was compared between control-sh and CBP-shA cell lines expressing HA-Ub (Fig. 2.3 C). In this case, the relative abundance of polyubiquitinated vs. mono-Ub/MUM p53 was quantitated showing an inverse correlation between the two forms when CBP was silenced, with polyubiquitination decreasing and mono-Ub/MUM forms increasing (Fig. 2.3 C). p300 and CBP are therefore both required to maintain physiologic levels

of polyubiquitinated p53 conjugates in unstressed U2OS cells.

The identity of ubiquitin chain linkages promoted by CBP was next tested by transfecting wt, K48-only or K63-only ubiquitin cDNAs into control or CBP-sh cells and immunoblotting p53 IPs of each lysate with HA antibody (Fig. 2.3 D). As might be expected based on the in vitro preference of p300 to catalyze K48-linked chains(54) which specifically catalyze proteasome degradation, CBP depletion resulted in the near total loss of K48-linked p53 polyubiquitination, but had no significant effect on barely detectable K63-linked p53-ubiquitin conjugates (Fig. 2.3 D).

Fig. 2.3. CBP and p300 both drive p53 polyubiquitination in vivo.

(A,B) U2OS cells were treated with the indicated siRNAs for 48 h and transfected with HA-Ub expression vector 24 h before harvest. MG132 (10 μM) was added for 4 h before harvest as indicated. Cell lysates were IP'd with anti-p53 Ab, followed by anti-HA or anti-p53 immunoblot. PolyUb indicates those p53 species >100 kDa that are larger than the largest MUM species as determined by use of non-chain-forming Ub moieties (54). Input lysates were blotted with the indicated antibodies. (C) CBP depletion modulates the pattern of p53 ubiquitination. Lysates of control-sh or CBP-shA cells expressing HA-Ub were immunoprecipitated with anti-p53 antibody and the IP's and input lysates immunoblotted with the indicated antibodies. The abundance of polyubiquitinated and mono-Ub/MUM species were quantitated by densitometry and plotted as a percentage of total ubiquitinated species from each cell line. (D) CBP regulates K48-linked p53 polyubiquitination. Control and CBP shRNA expressing cells were transfected with HA tagged wild type, K48-only and K63-only ubiquitin cDNAs. Lysates were immunoprecipitated with anti-p53 antibody, followed by HA and p53 immunoblot. The migration of polyubiquitinated p53 species is indicated. Lysates were also immunoblotted with HA antibody to demonstrate expression of exogenous ubiquitins, with HSP70 antibody as loading control, and CBP antibody to verify knockdown efficiency.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siCon
siCBP
sip300
Ub-HA
IP p53
WB HA
210KD
Poly-Ub-p53
110KD
71KD
WB: p53
Input
WB HA
WB HSP70
WB:CBP
WB:p300
B
siCon
siCBP
sip300
MG132 (10uM)
Ub-HA
IP p53
WB HA
210KD
Poly-Ub-p53
110KD
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Cont-sh
CBP-shA
HA-Ub
IP p53
WB HA
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Poly-Ub
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51KD
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WB HA
WB GAPDH
WB CBP
WB p300
Relative Ub signal
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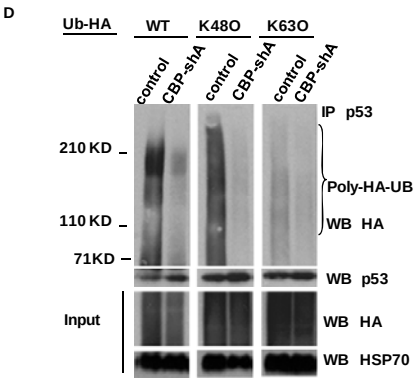
Cont-sh CBP-shA


D
Ub-HA
WT
K48O
K63O
control
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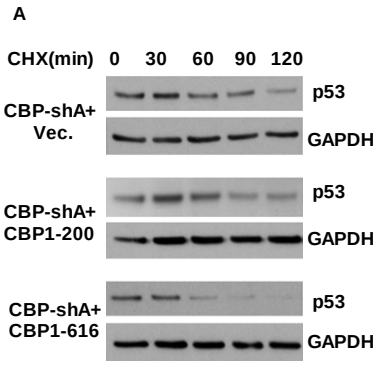
control
CBP-shA
control
CBP-shA
IP p53
210 KD
Poly-HA-UB
110 KD
WB HA
71KD
WB p53
Input
WB HA
WB HSP70

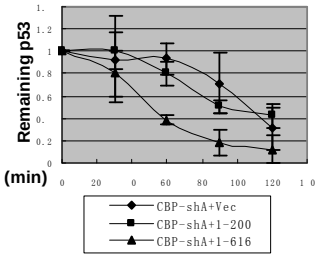
CBP N-terminal sequences destabilize p53.

To map the portion of CBP responsible for p53 destabilization, vector control, full-length or truncated CBP cDNAs were expressed as “rescue” alleles in CBP-deficient CBP-shA cells, and p53 stability was measured by cycloheximide-decay analysis. Full-length CBP* (mutated in the shRNA target sequence) expression in CBP-shA cells restored p53 half-life to the normal 60 min from the 120 min observed in CBP-shA cells (Fig. 2.4 C). Given that the p300 N terminus has been proposed as necessary and sufficient for E4 activity (residues 1–595) (54), the homologous portion of the CBP N terminus was analyzed in detail for its ability to rescue p53 instability in CBP-shA cells (Fig. 2.4 A). CBP (1–616) and (1–200), which do and do not, respectively, include the C/H1-TAZ1 domain (residues 348–432), were expressed in CBP-shA cells (Fig. 2.4.B). p53 was destabilized from a t1/2 of 110 min seen in CBP-shA cells to 55 min in cells expressing CBP (1—616) (Fig. 2.4 A), while the p53 t1/2 remained similar to the value in CBP-shA cells when CBP (1–200) was expressed (100 min)) (Fig. 2.4 A). Thus, the CBP N terminus is sufficient to destabilize p53, and the sequence between residues 200 and 616, including the C/H1-TAZ1 domain, is required for this activity.

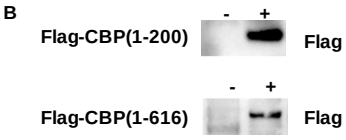
Fig. 2.4 The CBP N terminus regulates p53 stability.

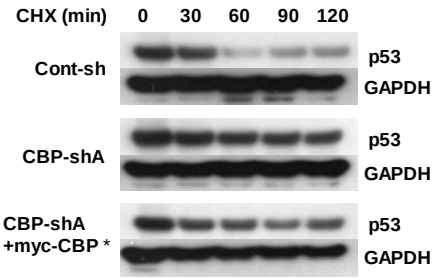
(A) CBP N-terminus regulates p53 stability. (Top) CBP-shA cells were transfected with vector or the indicated CBP rescue alleles, treated with cycloheximide 48 h after transfection, and lysates analyzed by p53 immunoblot. (Bottom) p53 levels were quantitated by densitometry, and half-life calculated based on decay of normalized (to GAPDH loading control) p53 levels to 50% of their original level. Values are an average of three independent experiments. Error bars, ±1 S.D. . (B) Expression of the indicated truncated FLAG-tagged CBP alleles used in Fig. 2.4 A was confirmed by anti-FLAG immunoblot. (C) Rescue of p53 instability by CBP in CBP-sh cells. (Top) The indicated shRNA-expressing cell lines were treated with cycloheximide, and lysates harvested at the indicated times and analyzed by p53 immunoblot. CBP* indicates that a silently mutated shRNA-resistant CBP cDNA was transfected into CBP-shA cells 48 h before cycloheximide treatment. (Middle) The expression of myc-tagged CBP* was evaluated by anti-myc immunoblot of the time 0 lysate. (Bottom) p53 abundance was quantitated at each time point, and t1/2 calculated after normalization for loading.

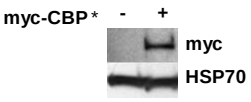


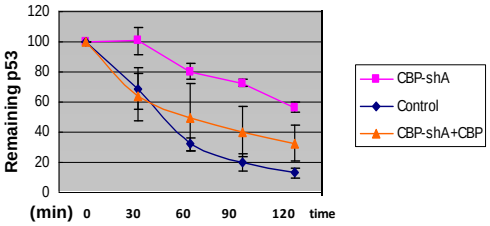


U2OS-sh lines	Half life (min)
CBP-shA+Vec	110
CBP-shA+CBP1-200	100
CBP-shA+CBP1-616	55









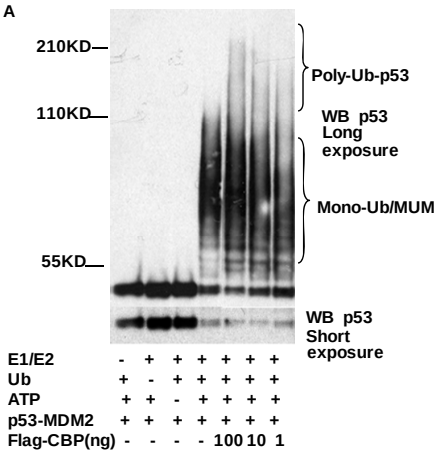
U2OS sh-line	Halflife (min)
Control	60
CBP-shA	>120
CBP-shA+CBP	60

The CBP N Terminus exhibits E4 activity in vitro and in vivo.

Given the suggestion from the in vivo data that CBP and p300 both exhibit an E4 function in vivo, we verified that CBP, like p300 (54), could act directly as an E4 in a reconstituted in vitro p53 ubiquitination reaction using purified components (Fig. 2.5 A). p53-MUM conjugates were prepared using purified E1, ubch5a (E2), Ub, and limiting quantities of purified recombinant MDM2 (54). When purified FLAG-CBP was added to this reaction, there was a dose-dependent increase in the abundance and the maximum molecular weight of high molecular weight species of p53, consistent with polyubiquitination (polyubiquitin conjugates are assumed at a molecular weight of p53 species >100 kDa) (54) (Fig. 2.5 A). Control reactions lacking MDM2 revealed no ubiquitination activity of p300 or CBP toward native p53 (Fig. 2.5 B, bottom), and eliminating p53 from the reaction eliminated all signal on the p53 immunoblots (Fig. 2. 5 B, top). To quantitate the E4 effect of CBP, the ratio of p53 signal in the high molecular weight range (>100 kDa) of the gel to total p53 signal was calculated at 20-kDa intervals between 100 kDa and the top of the gel, and plotted vs. mean molecular weight (Fig. 2.5 A, bottom). The clear separation of the control (MDM2-only) curve from the CBP titration curves reveals that CBP had a clearly quantifiable impact on Ub content and, by inference, Ub chain length of p53-Ub conjugates.

Fig. 2.5 Purified recombinant CBP protein catalyzes p53 polyubiquitination.

(A) (Top) Purified recombinant p53-MDM2 protein complexes were preincubated with ubiquitin reaction components at 37 °C for 30 min. Purified FLAG-CBP (1, 10, or 100 ng) was then added, followed by further incubation for 1 h at 37 °C. Reactions were analyzed by p53 immunoblot. A shorter exposure (middle) shows the unmodified p53 protein level. (Bottom) The ratio of high molecular weight (>100 kDa) p53 species: Total p53 abundance was plotted at 20-kDa intervals for the indicated conditions. (B) Control experiments for p53 ubiquitination (E4) assay in Fig. 2B. (Top) Purified HA-p53, FLAG-MDM2, FLAG-CBP, or FLAG-p300 as indicated were incubated in ubiquitination reactions containing the indicated components, and products were analyzed by western blot with p53 antibody. (Bottom) Purified HA-p53, FLAG-MDM2, FLAG-CBP, or FLAG-p300 were added as indicated to the indicated components, followed by additional incubation and western blot of the reaction with anti-p53 antibody.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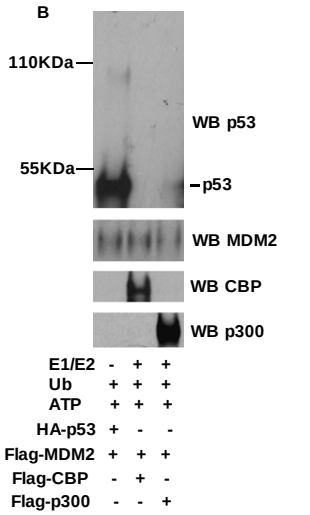
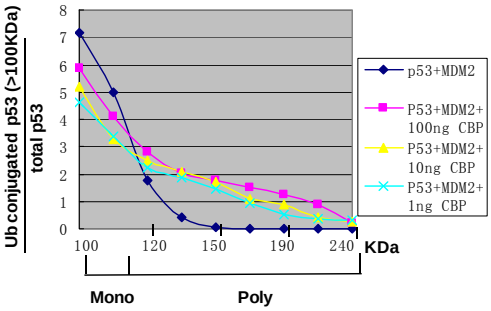
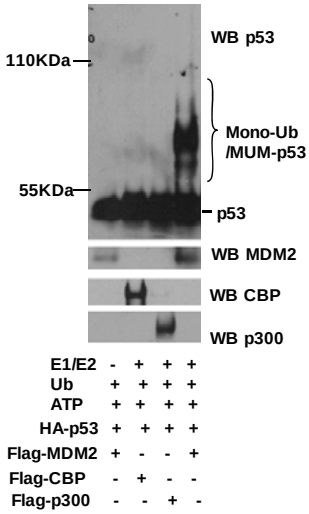
210KD
110KD
55KD
Poly-Ub-p53
WB p53
Long
exposure
Mono-Ub/MUM
WB p53
Short
exposure
E1/E2 - + + + + + +
Ub + - + + + + +
ATP + + - + + + +
p53-MDM2 + + + + + + +
Flag-CBP(ng) - - - - 100 10 1


B
110KDa
55KDa
WB p53
p53
WB MDM2
WB CBP
WB p300
E1/E2 - + +
Ub + + +
ATP + + +
HA-p53 + - -
Flag-MDM2 + + +
Flag-CBP - + -
Flag-p300 - - +

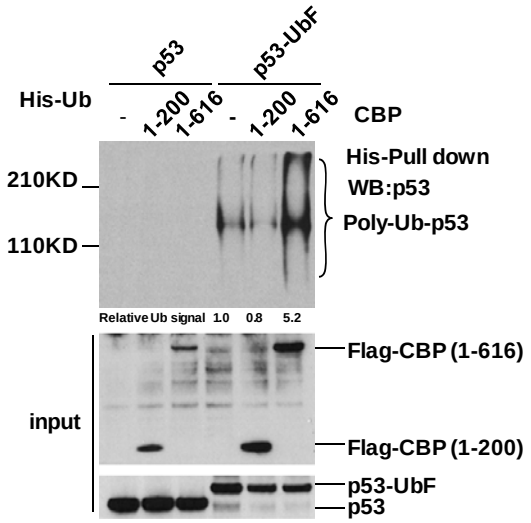

Ub conjugated p53 (>100KDa)
total p53
0
1
2
3
4
5
6
7
8
100
120
150
190
240
KDa
Mono
Poly
p53+MDM2
P53+MDM2+
100ng CBP
P53+MDM2+
10ng CBP
P53+MDM2+
1ng CBP


110KDa
55KDa
WB p53
Mono-Ub
/MUM-p53
p53
WB MDM2
WB CBP
WB p300
E1/E2 - + + +
Ub + + + +
ATP + + + +
HA-p53 + + + +
Flag-MDM2 + - - +
Flag-CBP - + - -
Flag-p300 - - + -

To demonstrate that the CBP N terminus could also act as an E4 for p53-mono-Ub or MUM conjugates in vivo, p53 or p53-Ub fusion (Ub cDNA fused in frame to the p53 C terminus; p53-UbF) protein cDNAs were expressed in cells, to determine if p53-UbF could serve as a model for p53-monoUb or MUM conjugates, and act as a direct substrate for the CBP E4 activity. His-Ub and CBP (1–200) and (1–616) polypeptides were expressed along with p53 or p53-UbF in H1299 cells, followed by Ni-NTA pulldown of lysates and p53 immunoblotting. Analysis of lysates used for Ni-NTA pulldown by FLAG and p53 immunoblot revealed even expression of CBP (1–200) and CBP (1–616) (Fig. 2.6, middle), as well as equivalent expression of p53 and p53-UbF (Fig. 2.6, bottom). Coexpression of the CBP N terminus with native p53, which is not even multiply monoubiquitinated in H1299 cells (Fig. 2.6 top, first lane) had no effect, as ubiquitinated p53 remained undetectable (Fig. 2.6 top, second and third lanes). Coexpression of CBP (1–616) with p53-UbF resulted in robust induction of high molecular weight Ub conjugates of the p53-Ub fusion, as compared with the effects of control (empty vector) or CBP (1–200) coexpression, both of which were associated with a similar background level of p53UbF ubiquitination signal (Fig. 2.6). The effect of the CBP N terminus on the ubiquitination status of the p53-Ub fusion specifically demonstrates that the first 616 aa of CBP are sufficient to polyubiquitinate p53-monoUb or MUM conjugates in vivo as well as in vitro.

Fig. 2.6 The CBP N terminus is an active E4 in vivo.

H1299 cells were transfected with His-Ub vector, p53 or p53-UbF expression vectors, along with the indicated CBP plasmids or empty vector. (Top) Forty-eight hours after transfection, lysates were purified on Ni-NTA beads, and eluted products immunoblotted with anti-p53 antibody. (Bottom) Input lysates were immunoblotted with anti-FLAG and p53 antibodies. The abundance of high molecular weight p53-Ub conjugates was determined by scanning densitometry and normalized to total amount of p53UbF in the input lysate. Experiment shown is representative of three independent experiments.

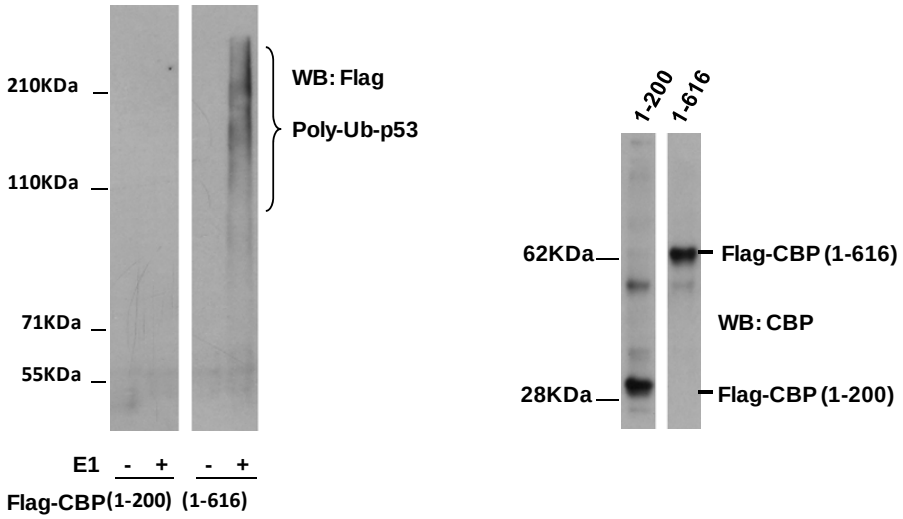


CBP E4 activity is separable from MDM2.

To definitively delineate the independent contribution of CBP to p53 polyubiquitination, a two-step in vitro E4 assay was developed. In the first step, p53-MUM conjugates were prepared by incubating with MDM2 in a similar fashion as seen in Fig. 2B, fourth lane, using purified FLAG-MDM2/HA-p53 complex from insect cells. The reaction products were immunopurified on anti-HA-conjugated beads under stringent (RIPA buffer) conditions to remove MDM2. CBP (1–616) or (1–200) polypeptides were then added to the immobilized HA-p53-Ub conjugates with fresh E1, E2, ATP, and FLAG-ubiquitin, followed by washing of the beads and blotting of the reaction products with anti-FLAG antibody. As is seen in Fig. 2.7, CBP (1–616), but not (1–200), clearly catalyzed the synthesis of polyubiquitin chains when p53 had been previously mono- or multiply monoubiquitinated by MDM2. Moreover, no FLAG-MDM2 signal was seen among the reaction products (absence of signal at 90-kDa section of blot; Fig. 2.7), confirming that MDM2 had been effectively removed before the addition of CBP polypeptides. Thus, CBP (1–616) encodes an MDM2-independent p53-directed E4 function.

Fig. 2.7 Sequential E4 assay.

(Left) Purified FLAG-MDM2/HA-p53 complexes were exposed to E1, E2, Ub, and ATP to generate mono-Ub/MUM p53. HA-p53-Ub conjugates were purified by anti-HA IP and stringent washing, and then exposed to E2 and ATP a second time with or without E1, along with FLAG-Ub and affinity purified CBP (1–616) or (1–200). After further washes, HA-p53-Ub conjugates were analyzed by anti-FLAG immunoblot.(Right) Immunoblot of purified CBP polypeptides used in the two-step E4 assay. pN8.FLAG-CBP (1–616) and (1–200) were transfected into U2OS cells, and FLAG-CBP (1–616) and (1–200) polypeptides were purified from cell lysates by FLAG affinity chromatography, followed by immunoblotting of the purified eluted proteins with anti-CBP antibody.

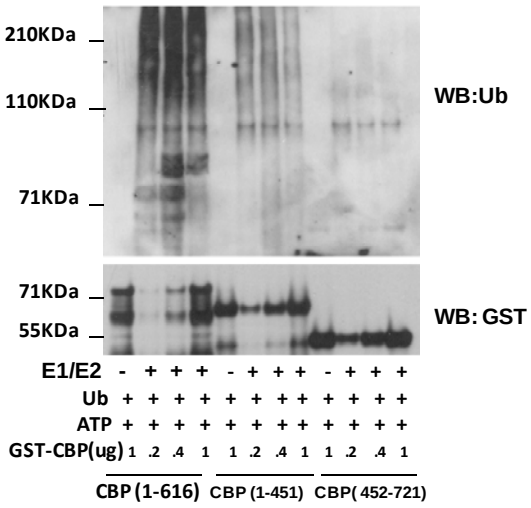


The CBP N terminus encodes an intrinsic E3 activity.

The E4 effect of CBP would be most consistent with an intrinsic MDM2-independent ubiquitin ligase activity that can only recognize a monoubiquitinated or multiply monoubiquitinated substrate, as has been observed for p300 (14). We investigated whether sequences arising from the CBP N terminus, which demonstrate E4 activity (Fig. 2.6, 2.7) and promote p53 instability in vivo (Fig. 2.4 A), encode an intrinsic E3 activity that could be clearly separated from MDM2. Prokaryotically synthesized, purified, GST-CBP (1–616), (1–451), and (452–721) were incubated with Ub, E1, E2 (ubch5a), and ATP in an autoubiquitination reaction, and the reaction products detected by anti-Ub immunoblot. Titration of GST-CBP (1–616) or (1–451) with standard ubiquitin reaction components revealed robust E3 activity (Fig. 2.8). GST-CBP (452–721), however, was inactive as an E3 (Fig. 2.8). Thus, the CBP polypeptide that promotes p53 degradation in vivo and p53 polyubiquitination in vivo and in vitro, also encodes an intrinsic, MDM2-independent, E3 ubiquitin ligase domain. The core sequences responsible for this activity appear to lie within at least the N-terminal 451 aa of CBP, and include the C/H1-TAZ1 domain.

Fig. 2.8 The CBP N terminus harbors intrinsic E3 activity.

(Upper) Purified GST-CBP fusion polypeptides were incubated with E1, ubch5a, ATP, and Ub for 1h at 37 °C followed by immunoblotting of the reaction products for Ub. (Lower) The immunoblot was reprobed with anti-GST antibody to reveal the abundance of the GST-CBP fusion proteins added to each reaction.



CBP promotes p53 degradation in the cytoplasm.

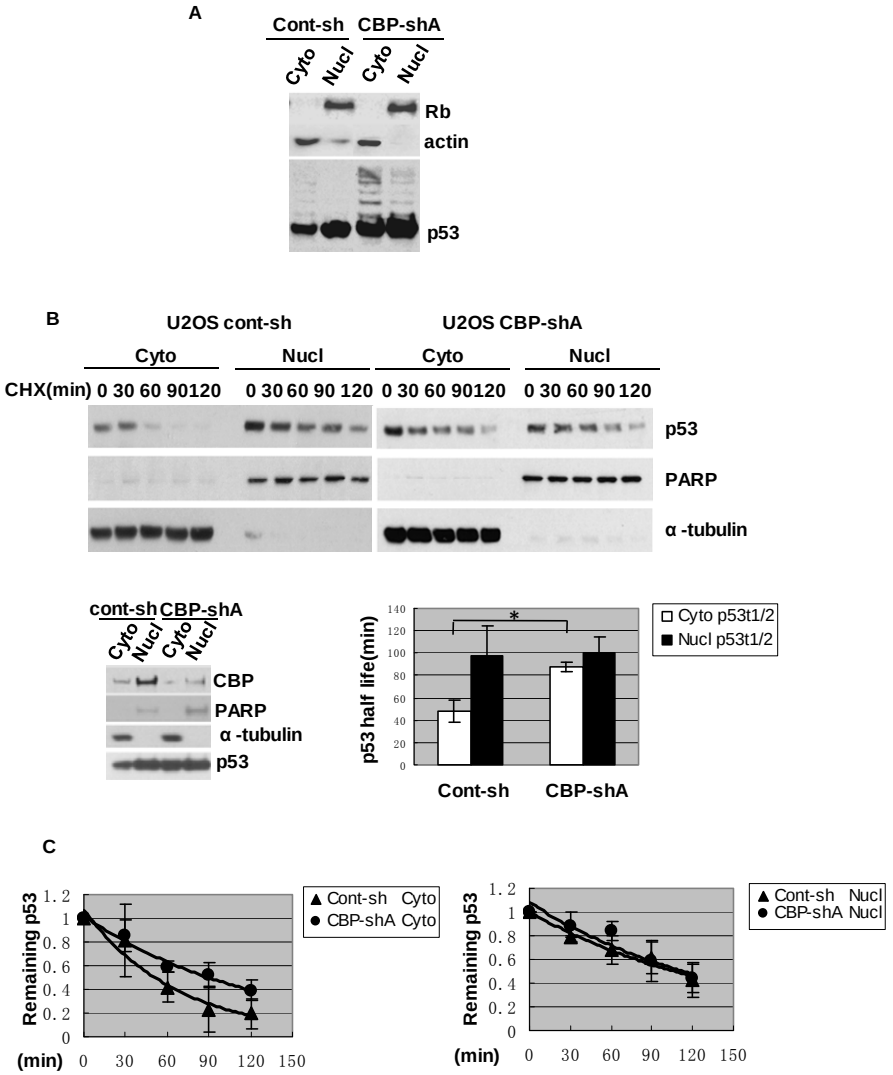
Since p300/CBP are considered to be nuclear coactivators (51), but p53 and MDM2 both shuttle between nucleus and cytoplasm (142), the cellular localization of CBP's regulation of p53 ubiquitination and degradation was explored further. Levels of unmodified p53 were proportionately increased in both nuclear and cytoplasmic fractions in CBP-shA vs. control cells, and controls for nuclear (Rb) and cytoplasmic (actin) proteins revealed that there was little cross-contamination of the fractions (Fig. 2.9A). Mono-Ub/MUM p53 was found predominantly in the cytoplasm, and levels of this species in the cytoplasm were significantly increased in CBP-shA vs. control cells, either due to the overall increase in total p53, or a loss of an (E4) activity required to convert p53-mono-Ub or p53-MUM conjugates to polyubiquitinated forms (Fig. 2.9A).

To determine if the differential ubiquitination of nuclear and cytoplasmic p53 correlated with different rates of turnover, nuclear and cytoplasmic p53 half-life was measured by cycloheximide decay in control and CBP-shA cells (Fig. 2.9 B and Fig. 2.9 C). PARP (nuclear marker) and α-tubulin (cytoplasmic marker) immunoblots of the fractions from each time point revealed negligible cross-contamination (Fig. 2.9 B). Surprisingly, CBP silencing led to stabilization of the normally rapidly degraded cytoplasmic p53 ($t_{1/2}$ increase from 50 to 85 min, $P = 0.01$), but had no significant effect on the slower turnover of nuclear p53 ($t_{1/2}$ increase from 95 to 100 min, $P = 0.63$; Fig. 2.9 B and Fig. 2.9 C). Given that p300/CBP are considered to be nuclear

proteins ((35, 90)), but the major effect of CBP depletion was on cytoplasmic p53 half-life, the possibility that cytoplasmic pools of CBP or p300 might influence p53 metabolism was explored.

Fig. 2.9 Subcellular localization of CBP/p300 E3 activity.

(A) p53 localization in CBP-deficient cells. Nuclear or cytoplasmic fractions of control or CBP-shA cells were analyzed by immunoblotting with Rb (nuclear marker), actin (cytoplasmic marker), and p53 antibodies. (B) CBP regulation of p53 turnover in the cytoplasm and nucleus. Control and CBP-shA U2OS cells treated with cycloheximide were fractionated into nuclear and cytoplasmic fractions at the indicated time points. (Top three panels) The fractions were immunoblotted for p53, PARP (nuclear marker), and actin (cytoplasmic marker). (Lower left) Subcellular fractions (time = 0) from control and CBP-shA cells were immunoblotted with anti-CBP, anti-PARP, anti-α-tubulin, and anti-p53 antibodies. (Lower right) Determination of nuclear and cytoplasmic p53 half-life from Cont-sh and CBP-shA cells. Result is the average half-life from four separate experiments. * indicates significant P = 0.01 for difference in cytoplasmic p53 t1/2 between Cont-sh and CBP-shA. (C) Regulation of cytoplasmic vs. nuclear p53 half-life by CBP. Decay curves of p53 abundance for the CHX-decay assay of nuclear vs. cytoplasmic p53 in control-sh vs. CBP-shA cells described in Fig. 2.9 B. Error bars,+/- 1 S.D.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CBP-shA
Cyto
Nucl
Cyto
Nucl
Rb
actin
p53
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U2OS cont-sh
U2OS CBP-shA
Cyto
Nucl
Cyto
Nucl
CHX(min) 0 30 60 90120 0 30 60 90 120 0 30 60 90120 0 30 60 90 120
p53
PARP
α -tubulin
cont-sh
CBP-shA
Cyto
Nucl
Cyto
Nucl
CBP
PARP
α -tubulin
p53
p53 half life(min)
140
120
100
80
60
40
20
0
*
Cyto p53t1/2
Nucl p53t1/2
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1.2
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(min) 0 30 60 90 120 150
Cont-sh Cyto
CBP-shA Cyto
Remaining p53
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0.8
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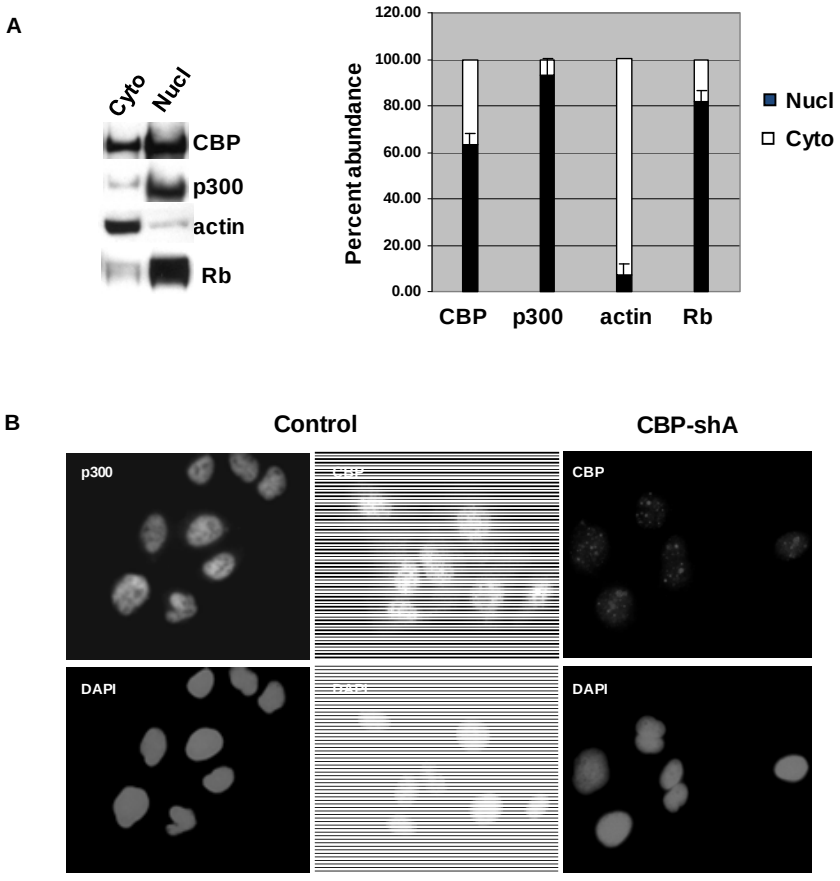
(min) 0 30 60 90 120 150
Cont-sh Nucl
CBP-shA Nucl

CBP/p300 are partly cytoplasmic.

CBP and p300 are localized in PML oncogenic domains (PODs) and senescence-associated nuclear bodies, respectively ((90, 120)). To gain a more precise understanding of p300 and CBP subcellular localization, their abundance in cytoplasmic and nuclear fractions of U2OS cells was determined (Fig. 2.10 A). The nuclear:cytoplasmic abundance ratios of Rb and actin, as determined by densitometry, revealed that the upper limit of cross-contamination of one fraction into the other was no greater than 20% in either direction (Fig. 2.10.A). CBP and p300 were predominantly nuclear, as expected, but for CBP especially, a clearly detectable quantity was found in the cytosolic fraction. Moreover, a much higher fraction of CBP was cytosolic (40%), compared with p300 (5%) (Fig. 2.10A). Stoichiometrically, however, the total amount of cellular cytosolic p300 and CBP may be similar, as the total cellular abundance of p300 is ~10-fold greater than that of CBP, as determined by calibrated immunoblotting of the two proteins from cell lysates (D. Shi, S.R. Grossman, unpublished observations). These fractionation results were further confirmed by immunofluorescent staining of U2OS cells for CBP and p300, where a clear cytoplasmic signal was detected for CBP, but p300 appeared predominantly nuclear (Fig. 2.10B). The specificity of the CBP immunofluorescence signal was confirmed by the near complete loss of CBP staining seen in CBP-shA cells (Fig.2.10B).

Fig 2.10 Subcellular localization of p300 and CBP.

(A)U2OS cells were fractionated into nuclear and cytoplasmic fractions, and the fractions were immunoblotted for CBP, p300, Rb (nuclear marker), and actin (cytoplasmic marker). Protein abundances were quantitated from 3 separate experiments by densitometry and average values calculated. Error bars indicate +/- 1 S.D. (B) Immunofluorescence analysis of p300 and CBP subcellular localization. Control or CBP-shA cells were stained with DAPI or antibodies against CBP or p300, followed by FITC-conjugated anti-IgG. Photomicrographs of p300 and CBP staining in all panels were taken at the same magnification (600) and exposure.



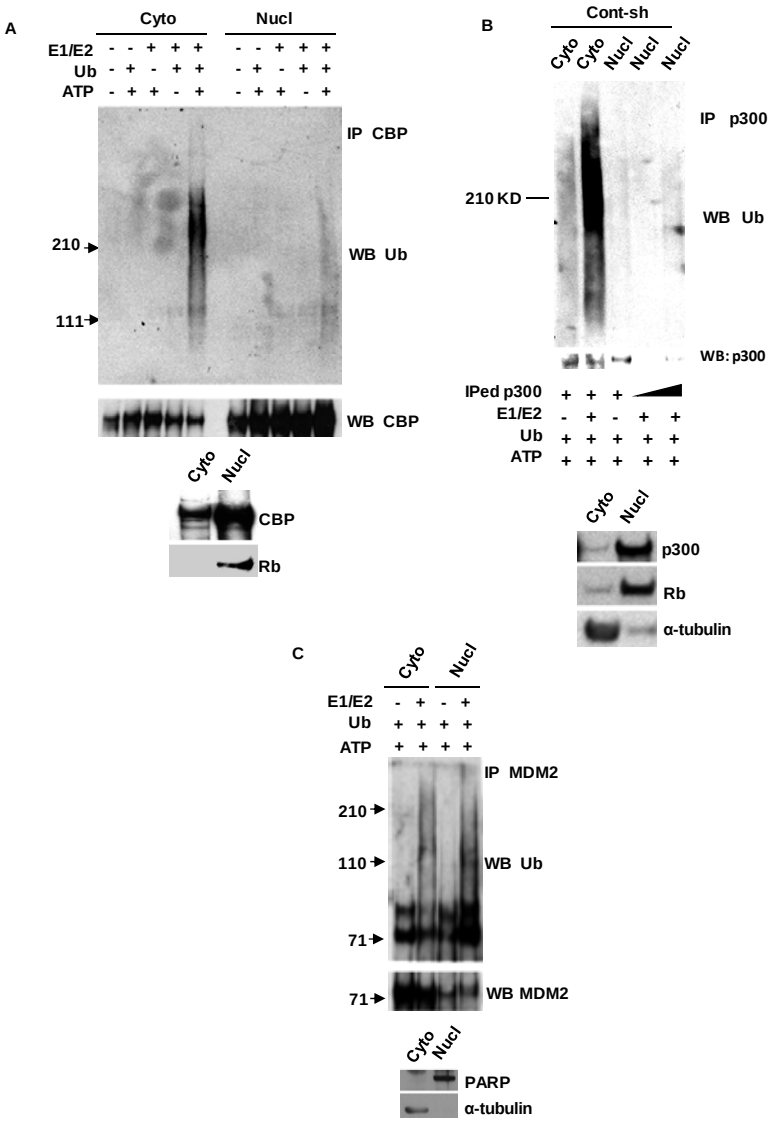
CBP/p300 E3 activities are exclusively cytoplasmic.

To determine if nuclear and cytoplasmic CBP or p300 molecules have any differential capacities to influence p53 ubiquitination, nuclear and cytoplasmic CBP/p300 were assayed for E3 autoubiquitination activity. Nuclear and cytoplasmic fractions of U2OS cells were IP'd with anti-CBP or p300 antibody, and the IPs were assayed for E3 activity by the addition of ubiquitin, E1, E2 (ubch5a), and ATP, followed by Ub, CBP, or p300 immunoblotting (Fig. 2.11A and Fig. 2.11B). Dropout control reactions lacking E1/E2, Ub, or ATP were also performed to demonstrate that any Ub chains observed were the result of bona fide in vitro E3 activity and not contamination by cellular Ub chains binding nonspecifically to the IPs (Fig. 2.11A and Fig. 2.11B). CBP and p300 were observed in the cytoplasm and nuclear fractions as expected, and controls indicated little cross-contamination (Fig. 2.11A, bottom, and Fig. 2.11B, bottom). Surprisingly, CBP and p300 derived from cytoplasm demonstrated robust E3 autoubiquitination activity dependent on the simultaneous addition of E1/E2, Ub, and ATP, whereas nuclear-derived CBP/p300 exhibited much lower or undetectable activity (Fig. 2.11A and Fig. 2.11B). CBP/p300 immunoblotting of the reactions revealed that the lack of E3 activity associated with nuclear CBP/p300 could not be explained by lesser abundance (Fig. 2.11A and Fig. 2.11B). The lack of nuclear CBP autoubiquitination activity could also not be explained by nonspecific inhibition by extraction conditions or inhibitory factors within the nuclear fraction, as immunopurified nuclear MDM2 exhibited easily detected autoubiquitination activity,

which if anything, had a higher specific activity than cytoplasmic MDM2, based on the relative abundance of MDM2 in the two fractions (Fig. 2.11C).

Fig. 2.11 p300/CBP E3 activities are predominantly cytoplasmic.

(A) Cytoplasmic CBP is associated with E3 ligase activity. (Top and Middle) Nuclear and cytoplasmic fractions of U2OS cells were immunoprecipitated with anti-CBP antibody, and the washed IPs incubated with E1/E2, Ub, and ATP as indicated, followed by anti-Ub and CBP immunoblotting of the reactions. (Bottom) Immunoblot of CBP and Rb (nuclear marker) in nuclear and cytoplasmic fractions. (B) Cytoplasmic p300 is associated with E3 ligase activity. (Upper) Nuclear and cytoplasmic fractions of control-sh U2OS cells were immunoprecipitated with anti-p300 antibody, and the washed IPs were incubated with E1/E2, Ub, and ATP as indicated, followed by Ub and p300 immunoblotting of the reactions. (Lower) Immunoblot of p300, Rb (nuclear marker), and -tubulin (cytosolic marker) in the nuclear and cytoplamic fractions of control-sh cells. (C) Nuclear and cytoplasmic MDM2 E3 activity. Cytoplasmic and nuclear fractions were IP'd with anti-MDM2 antibody, and the IPs incubated with E1/E2, Ub, and ATP, followed by anti-Ub (top) or anti-MDM2 (middle) immunoblotting of the reactions. The subcellular fractions were immunoblotted with anti-tubulin and PARP antibodies (bottom) to confirm the lack of cross-contamination of the fractions.


A
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ATP
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Nucl
p300
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Nucl
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ATP
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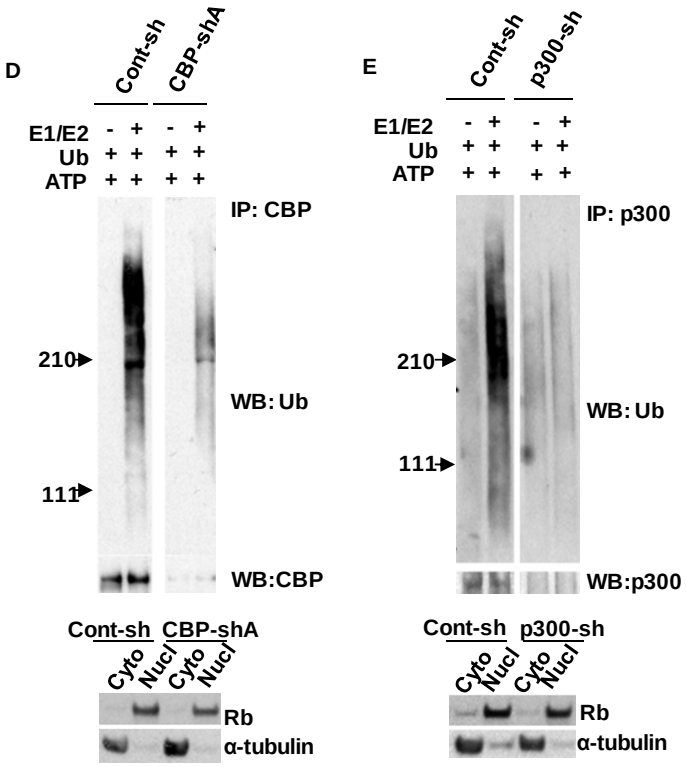
WB Ub
71
WB MDM2
Cyto
Nucl
PARP
α-tubulin

To further assess the specific dependence of the observed E3 activity on the actual presence of CBP or p300 in the reaction, cytoplasmic fractions of control, CBP-shA, and p300-sh cells were IP'd with anti-CBP or anti-p300 antibodies, as appropriate, and the IPs assayed for E3 activity with or without added E1 and E2 (Fig. 2.11D and 2.11E). There was no significant cross-contamination of nuclear and cytoplasmic markers (Fig. 2.11D and E, bottom), and the IPs were also probed for p300 and CBP to assure that the levels of p300/CBP in the IPs reflected the respective shRNA knockdowns for each (Fig. 2.11D and E, middle). CBP and p300 IPs from control cell cytoplasm were both highly enriched for E3 autoubiquitination activity dependent on added E1/E2 (Fig. 2.11D and E, upper). However, CBP and p300 IPs from both CBP-shA and p300-sh cytoplasm were substantially depleted of E3 activity (Fig. 2.11D and E, upper), confirming that the E3 activity seen in the p300 and CBP IPs from control cell cytoplasm did depend on the specific presence of p300 or CBP proteins in the respective reactions.

Fig. 2.11 p300/CBP E3 activities are predominantly cytoplasmic.

(D and E) (Top and middle) Cytoplasmic fractions of control, CBP-shA, or p300-sh cells as indicated were immunoprecipitated with anti-p300 or anti-CBP antibodies as indicated. After washing, the IPs were assayed for E3 activity by incubating with E1, E2, ATP, and ubiquitin as indicated, followed by anti-CBP, p300, and ubiquitin immunoblotting. (Bottom) Cytoplasmic fractions of the indicated cell lines were immunoblotted with anti-Rb (nuclear marker) and anti-tubulin (cytoplasmic marker) antibodies.

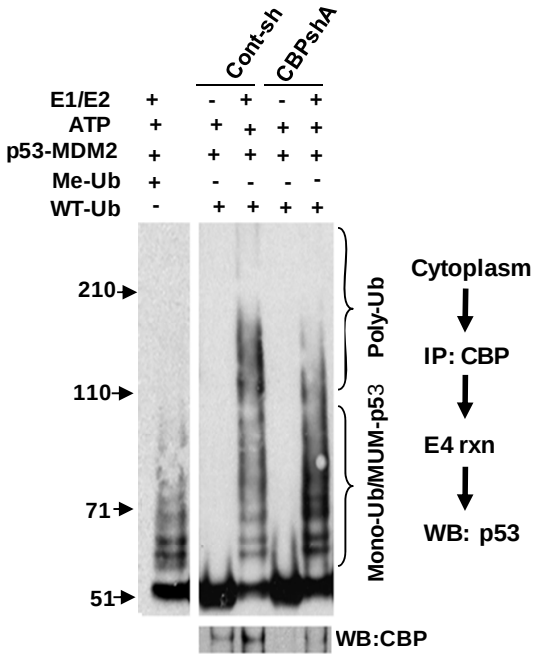


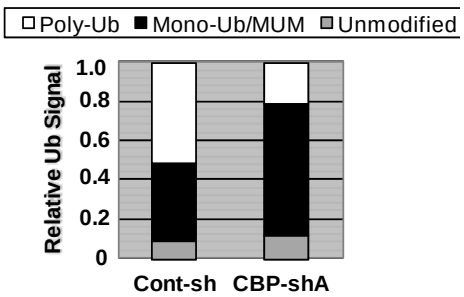
Cytoplasmic CBP is an active p53 E4.

The specific localization of E3 autoubiquitination activity of CBP/p300 to the cytoplasm suggests that their E4 activity, likewise resides in the cytoplasm. To confirm that cytoplasmic CBP was an active E4 for p53, immunopurified cytoplasmic CBP derived from control-sh or CBP-shA cells was exposed to MDM2/p53 complexes in the presence or absence of E1/E2, along with Ub and ATP (Fig. 2.11F). To identify the migration positions of p53-mono-Ub/MUM species, p53/MDM2 complexes were incubated with E1/E2, methyl-Ub (nonconjugatable), and ATP (Fig.2.11F, first lane). CBP IP'd from control-sh cells clearly catalyzed the formation of polyubiquitinated p53 (p53-polyubiquitin conjugates ≈50% of total p53 species; Fig. 2.11F), and this activity was significantly reduced in proportion to the reduction in CBP abundance when a CBP IP from CBP-shA cells was used as the source for cytoplasmic CBP (p53-polyubiquitin conjugates ≈20% of total p53 species; Fig. 2.11F). Thus, cytoplasmic CBP is an active E4 for previously mono/multiply monoubiquitinated p53.

Fig. 2.11 F E4 activity of cytoplasmic CBP.

p53/MDM2 complexes (insect-cell-derived) were incubated with E1/E2, Ub, or methyl-Ub, ATP and CBP IPs from control-sh or CBP-shA cytoplasmic fractions as indicated, followed by anti-p53 (Top) or anti-CBP (Middle) immunoblotting of the reactions. Migration positions for native, monoubiquitinated/MUM and polyubiquitinated p53 species are indicated. (Bottom) Relative abundances of native, mono-Ub/MUM, and polyubiquitinated p53 species were quantitated by densitometry.





2.4 Discussion

p300 and its paralog CBP have been identified as physiological regulators of p53 ubiquitination and stability. Depletion of CBP or p300 resulted in p53 stabilization, and CBP and p300 were both required for physiologic p53 polyubiquitination. The CBP N terminus (1–616) was found to encode a p53 destabilization function in vivo, and residues 1–616 and 1–451, respectively, constitute minimal functional E4 and E3 domains within CBP. CBP and p300 E3 activities were only observed from cytoplasmic fractions, and p53-mono-Ub and MUM conjugates accumulated in the cytoplasm of CBP silenced cells. The degradation of cytoplasmic p53 was specifically altered by CBP silencing, consistent with the cytoplasmic localization of CBP E4 function in vivo.

The N terminus of p300 harbors its E3/E4 activity (54), and the same region of CBP encodes its E3/E4 activity and shares significant areas of sequence conservation ((3, 102)). Within this region of p300/CBP is a cys-his-rich region, but no signatures for RING, HECT, U-box, or PHD domains can be found. Based on findings with the A20, Rabex-5, and E4F1 proteins ((92, 106, 149)), a generic Zn2+-binding region (cys- or cys-his-rich) can encode an active E3, with no clear sequence or structural homology to the Zn2+-binding RING E3 domain. Perhaps more directly relevant, the evolutionarily related HAT P/CAF was recently shown to encode a noncanonical E3 that targets MDM2 (98). In this case, the protein domain responsible for the activity

contains no structural motifs found in known E3 proteins (98).

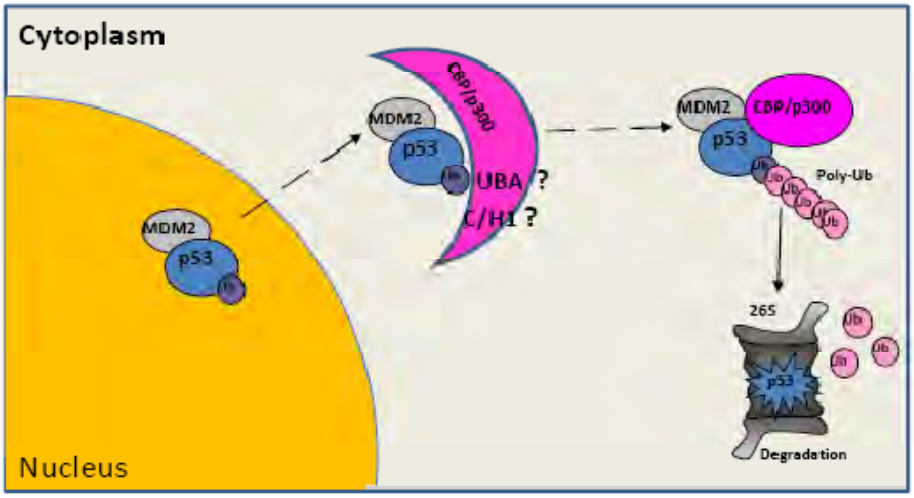
Subcellular fractionation revealed that p300/CBP E3 activities are limited to the cytoplasm and presumably account for the activity that converts mono-Ub/MUM p53 conjugates to polyubiquitinated unstable p53 in the cytoplasm. The mechanism of localization of p300/CBP E3 activity to the cytoplasm cannot yet be explained. Either a cytoplasmic-specific modification of p300/CBP or p300/CBP-interacting factor activates the activity or an interacting factor or modification in the nucleus represses it. The spatial separation of p300/CBP's E3/E4 activity from their nuclear HAT functions is intriguing and solves the seeming paradox of how these two presumed opposing regulatory functions exist within the same molecules (53).

This work raises a number of questions critical to p53, ubiquitin/proteasome, and cancer biology. How the plethora of p53 E3s, and now E4s, are coordinated to achieve p53 homeostasis remains unclear, although it is likely that the subcellular localization of these enzymes will be of critical importance to understanding their roles. The biochemical mechanism by which CBP/p300 recognizes ubiquitinated, and not native, p53 for further ubiquitination is also presently not understood. The simplest explanation for the preferred recognition of ubiquitinated substrate would be the existence of a Ub recognition motif within the p300/CBP N-termini—as is seen in the Rabex-5 atypical E3 (106), although no such domain is readily recognized in p300/CBP by homology search. Finally, the implications of these findings for cancer

biology and therapy are potentially significant. As MDM2 is now a bona fide clinically relevant drug target for cancer therapy ((146)), other enzymes in the p53 stability regulation pathway, such as the p300/CBP E4 domain, might also be effectively targeted in human cancers.

Fig. 2.12 CBP /p300 are cytoplasmic E4 ubiquitin ligases of p53

CBP/p300 are the physiological E4 ubiquitin ligases of p53 and their ubiquitin ligase activity resides in the cytoplasm. E4 ubiquitin ligase activity is separable from MDM2; polyubiquitination of p53 depends upon MDM2 E3 mono-ub activity as the priming step. We hypothesize that E4 enzymatic activity is contained in the C/H1 domain and a potential UBA (ubiquitin association domain) domain is required for recognizing only mono-ub-p53, but not unmodified p53.



2.5 Materials and Methods

Cell Culture and Plasmids

U2OS cells were grown in DMEM supplemented with 10% FBS and antibiotics. Cells were treated, where noted, with 100 μg/mL cycloheximide, 2 μM Dox, or 10 μM MG-132 (Sigma). Plasmid transfection was done with Fugene 6 (Roche), and siRNA transfections used Oligofectamine (Invitrogen). pRSV-CBPmyc ((79)) and pcDNA-UbHA (9) have been described. To generate pN8.Flag-CBP (1–616) and -CBP (1–200), CBP fragments were PCR-amplified from pRSV-CBPmyc and cloned into pN8.Flag vector using BamHI and EcoR1. An shRNA-resistant CBP allele (pRSV-CBP*myc) was generated by silent mutation of the shRNA target sequence (AACTCCAATAGC mutated to AATAGTAACTCT; CBP residues 190–193) within pRSV-CBPmyc. pGEX-CBP (1–616) and (1–200) were generated by cloning the indicated PCR-amplified fragments into pGEX-2tk. pCDNA3-p53-UbF, which has the Ub cDNA cloned in-frame to the 3′ end of the p53 cDNA, was the generous gift of Christine Blattner, and MT107 His-Ub expression vector has been described ((144)).

Western Blotting, Immunofluorescence, and Immunoprecipitation.

For western blot analyses, cells were lysed in cold NETN240 buffer (20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 10 μM $ZnCl_2$, 240 mM NaCl, 0.2% Triton-X 100), supplemented with complete EDTA-free protease inhibitor tablets (Roche). For immunoprecipitation of whole cell lysates, cells were lysed in cold RIPA buffer [50

mM Tris-HCl, pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1% Triton X-100, 0.5% DOC supplemented with fresh 5 mM NEM and complete EDTA-free tablets (Roche)]. Immunoprecipitations from whole cell lysates were performed in the lysis buffer overnight, followed by capture with Protein A agarose (Upstate) and five washes in lysis buffer. Western blot signals were quantified after visualization of primary antibody by HRP-conjugated secondary antibody and enhanced chemiluminescence or by fluorescent-labeled secondary antibody and detection by Odyssey blot scanner (LiCor), using ImageJ National Institutes of Health (NIH) software.

In Vitro E3 Assays of CBP/p300 or MDM2 E3 ligase activity

CBP, p300, or MDM2 were immunoprecipitated from 0.7 mg cytoplasm or 0.2 mg nuclear fractions diluted with high salt buffer [10 mM HEPES (pH 7.5), 150 mM NaCl, 150 mM KCl, 1 mM $MgCl_2$, 0.5% Triton X-100, supplemented with fresh 5 mM NEM and protease inhibitors) using A-22, N-15, or D-7 antibodies followed by protein A Sepharose. The IPs were washed in high salt buffer three times followed by two washes in Ub buffer (25 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Triton X-100, 0.5 mM DTT, 3 mM Mg-ATP). The washed and equilibrated IPs were then incubated with 100 ng E1 (rabbit; Boston Biochem), 25 ng E2 (UbcH5a, human recombinant; Boston Biochem), and 5 μg Ub (human recombinant; Boston Biochem) for 60 min at 37 °C. Reactions were then stopped by the addition of sample buffer,

followed by SDS-PAGE and immunoblotting. E3 assays using purified GST-CBP polypeptides were performed as described (14). GST-CBP (1–616), (1–451), and (452–721) (36) were expressed in BL21 cells and purified using glutathione-Sepharose (GE Healthcare). Purified GST-CBP (0.1, 0.2 or 0.5 μg) (1–616), (1–451), or (452–721) proteins were added to ubiquitin reaction components as indicated and incubated at 37 °C for 60 min, followed by analysis of the reaction products with anti-Ub antibody.

One-Step E4 Assay to measure CBP E4 ligase activity

Iinsect-cell-derived human p53 and FLAG-MDM2 were purified as a complex using FLAG M2 (Sigma) agarose as described (14), then incubated with ubiquitin reaction components (100 ng E1, rabbit; Boston Biochem), 25 ng E2 (UbcH5a, human recombinant; Boston Biochem), and 5 ng Ub (human recombinant; Boston Biochem) at 37 °C for 30 min. FLAG-CBP (1, 10, or 100 μg) immunopurified from baculovirus-infected Sf9 insect cells (with anti-FLAG resin/FLAG peptide elution) (35) or CBP IP'd from U2OS cytoplasmic fraction (see In Vitro E3 Assay section) was then added, followed by further incubation of the reaction at 37 °C for 60 min and p53 immunoblot of the reaction products.

Two-Step E4 Assay to measure E4 ligase activity of CBP

A two-step ubiquitination reaction was performed. First, insect-cell-derived p53 was

ubiquitinated by MDM2 as described above. To remove MDM2 and nontagged ubiquitin, p53 was then immunoprecipitated and washed three times with RIPA buffer, two times with PBS, and two times with Ub buffer. Immobilized p53 was then mixed with second ubiquitination reaction components [100 ng E1, 25 ng UbcH5a, 5 μg Flag-tagged Ub (Boston Biochem)] along with affinity-purified Flag-CBP (1–200) or (1–616) polypeptides obtained from transfected U2OS cells and incubated at 37 °C for 60 min. To remove CBP autoubiquitination products, beads were washed three times with RIPA buffer. p53 was eluted by boiling with NuPAGE LDS sample buffer, separated by SDS-PAGE, and visualized by immunoblotting with anti-Flag antibodies.

Generation of Knockdown Cell Lines.

Stable hairpin RNA (shRNA)-expressing cell lines were generated using the following shRNA target sequences: CATAAACAACTGTCGGAGC (CBP-shA), TAGTAACTCTGGCCATAGC (CBP-shB), TCATTTCACACTGGAAGAA (p300-sh). The CBP short hairpin oligonucleotides were cloned into pSuperior.puro (Oligoengine) using BglII/XhoI cloning sites. After transfection of the hairpin constructs, the cells were subjected to selection with 1 g/mL puromycin (AG Scientific). Independent clones were selected and evaluated for silencing by immunoblot. Antibodies: p53-DO1 (blotting), p53-FL393R (IP), actin C-2, Ub P4D1, CBP A22, HSP70 W27, MDM2 N-20 (blotting, Fig. S1A), MDM2 D-7 (IP) (all Santa Cruz Biotechnology), MDM2Ab-5 (blotting, Fig. S4B) (Calbiochem), GAPDH 6C5

(Advanced Immunochemical), myc-tag 4A6, p300 RW128 (Upstate), Rb (BD PharMingen), and phospho-p53 (Ser-15; Cell Signaling).

siRNA Transfection.

siRNA duplex corresponding to

CBP (forward: 5-AAUCCACAGUACCGAGAAAUGUU-3;

reverse: 5-CAUUUCUCGGUACUGUGGAUUUU-3),

p300 (forward: 5-CAGAGCAGUCCUGGAUUAGtt-3;

reverse: 5- CUAAUCCAGGACUGCUCUGtt-3),

Control (siGFP) were synthesized by Dharmacon. U2OS cells (1 107) were

transfected with 0.2 pmol siRNA using Oligofectamine (Invitrogen). Seventy-two hours after transfection, cells were harvested and analyzed for the expression level of CBP, p300, GAPDH, and p53 by western blotting.

Ni-NTA Pulldown of His-Tagged Ub Conjugates.

Cells were washed with ice-cold PBS and lysed in 7 mL guanidinium lysis buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris- HCl, pH 8.0, 5 mM imidazole, 10 mM mercaptoethanol). HisLink Protein Purification Resin (75 L; Promega) was added to the lysate, and the mixture was incubated by end-over-end

rotation at 4 °C for 16 h. The beads were successively washed with guanidinium buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH 8.0, 10 mM mercaptoethanol), buffer A (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH 6.3, 10 mM mercaptoethanol), buffer A plus 0.2% Triton X-100, and buffer A plus 0.1% Triton X-100. Ubiquitinated proteins were eluted with 200 mM imidazole in Ubiquitin Elution Buffer (5% SDS, 30% glycerol, 0.72 M mercaptoethanol, 0.15 M Tris-HCl, pH 6.7). The eluate was supplemented with NuPAGE LDS sample buffer and subjected to SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with the anti-p53 antibody DO-1 to detect ubiquitinated p53.

Subcellular Fractionation.

Cytoplasmic extracts were prepared by Dounce homogenization of cells (Type A pestle, 40 strokes) using low salt buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 1mM $MgCl_2$,15% glycerol supplemented with fresh 1 mM DTT, 5 mM NEM, and protease inhibitors), followed by low speed pelleting of nuclei (1,000 rpmfor 5 min). The supernatant of the low speed spin was centrifuged at high speed (14,000 rpm for 10 min) to remove membranes, and the supernatant was removed for use as cytoplasm. Nuclei pelleted from the low speed spin were washed three times in low salt buffer and then extracted with high salt buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 150 mM KCl, 1 mM $MgCl_2$, 0.5% Triton X-100 supplemented with fresh 5 mM NEM and

protease inhibitors) for 30min at 4 °C. The nuclear extract was then clarified by centrifugation (14,000 rpm for 15 min).

Chapter 3: Differential regulation of p53 by p300 and CBP upon DNA damage

3.1 Abstract

p300 and CREB-Binding Protein (CBP) act as multifunctional regulators of p53 via acetylase and ubiquitin ligase activities. The physiologic roles of the p300 and CBP coactivators in p53 regulation remain unclear as most prior work has utilized overexpression approaches. Analysis of p300 or CBP-deficient cells revealed that CBP and p300 exhibited differential regulation of p53 gene target expression, C-terminal acetylation, and biologic response after DNA damage. Neither p300 or CBP were specifically required for p21 induction after ionizing radiation. In contrast, p300 activated, and CBP repressed, PUMA expression after DNA damage, correlating respectively with acetylation of p53 C-terminal lysines (including K382) that required p300, and acetylation of p53 K320 that required CBP. Consistent with their gene expression effects, CBP deficiency augmented, and p300 deficiency blocked, apoptosis after doxorubicin treatment. The defect in p53 lysine-320 acetylation after DNA damage in CBP deficient cells was correlated with lower levels of the putative K320 acetylase P/CAF, but restoration of P/CAF levels in CBP deficient cells did not restore p53 K320 acetylation, nor did it attenuate excess apoptosis induced by CBP deficiency. CBP expression, however, did rescue both the K-320 acetylation and apoptosis phenotypes. CBP and p300 thus act in opposition after DNA damage, with

CBP directly or indirectly responsible for p53 K320 acetylation and PUMA repression, counterbalancing the apoptotic drive induced by p300 acetylation of p53 C-terminal lysines and its coactivation of PUMA expression.

3.2 Introduction

p53, or a component of its tumor suppressor pathway, is mutated or dysregulated in nearly all human cancers (108). Depending on yet unknown factors, it can signal cells to arrest, senesce or apoptose through both transcription-dependent as well as independent mechanisms (110, 134). Its activity is controlled at a post-translational level by a multitude of post-translational modifications, including phosphorylation, ubiquitination, sumoylation, neddylation, methylation and acetylation (9, 111). Of these, phosphorylation and acetylation are generally considered to be positively acting modifications, stimulated by cellular stress, and linked to increased protein stability and transcriptional activity (2, 12).

p300 and CBP are most widely known as coactivators encoding histone and non-histone acetyltyransferase activity. In vitro or overexpression studies have shown that p53 is acetylated by p300 or CBP (in vitro or by overexpression) at 5 separate C-terminal lysines (K370/K372/K373/K381/K382). Changes in charge or conformation induced by these modifications increase the affinity of p53 for its specific DNA target sequence (103). Additional lysines are targeted by other acetyltransferases, notably P/CAF (K320) (101, 125) and Tip60 (K120) (139, 140). Stress generally enhances

p53 acetylation in vivo (9), and acetylation of all of the abovementioned residues has been linked to regulation of the critical arrest/apoptosis decision of the p53 stress response (83, 139, 140). However, germ-line mutation of the C-terminal cluster of lysines acetylated by p300/CBP in mouse p53 did not greatly alter p53 stress responses (86), whereas mutation of the putative P/CAF target lysine (K317 in mouse) led to exaggerated apoptotic responses, suggesting a critical role for this lysine in regulating p53 function (18). Since P/CAF knockout cells or mice are phenotypically normal, and do not exhibit enhanced apoptosis (153), it remains unclear as to whether P/CAF is the physiologic K320 acetylase.

To better define the role of p300 or CBP in p53 regulation, p53 stability and function was studied in p300 or CBP deficient cells. CBP loss sensitized cells to doxorubicin-induced apoptosis, while p300 loss suppressed apoptosis after Dox. These functional outcomes correlated with differential effects of p300 and CBP loss on p53 C-terminal/K320 acetylation and p53-dependent gene expression after DNA damage. P300 and CBP therefore engage in complex and differential regulation of p53 that is required for its proper homeostasis in basal and stressed conditions.

3.3 Result

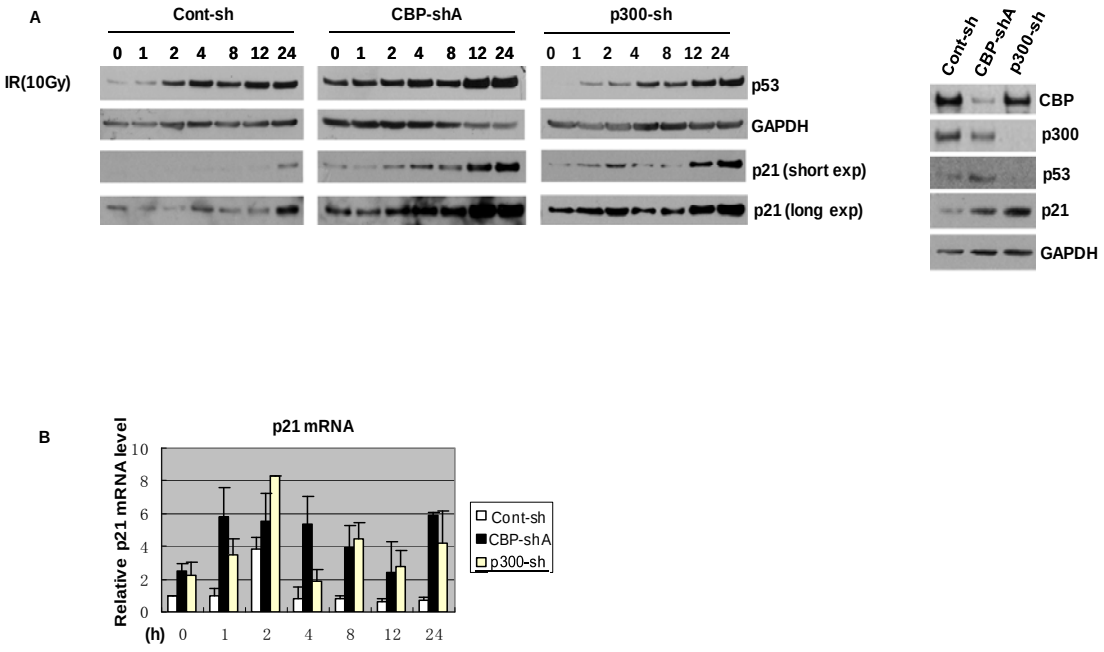
p300 and CBP are dispensable for p21 induction after gamma irradiation

Despite recent evidence that p300/CBP are negative regulators of p53 stability and abundance in unstressed cells (113, 129), both have previously been identified as

coactivators of p53 under conditions of cellular stress (6, 57, 97, 128). To determine whether the putative coactivation functions of p300/CBP supersede their ubiquitination functions under stress conditions, p300 or CBP-deficient U2OS osteosarcoma cells were exposed to 10 Gy IR and levels of p53 and p21 protein and mRNA measured over a 24 hr time course. p21 protein and mRNA were both induced after IR as expected in control cells, but surprisingly, augmented, and more rapid, p21 induction was observed in CBP or p300-deficient cells at both the protein and mRNA levels (Fig. 3.1 A, B). Thus, individually, physiologic levels of neither p300, nor CBP, are required for p21 transcriptional induction after IR in p53-competent U2OS cells.

Fig. 3.1 p21 and p53 expression profile in IR-treated p300/CBP deficient cells.

(left 3 panels) Control, CBP-shA or p300-sh cells were treated with IR (10 Gy) and lysates prepared at the indicated times and immunoblotted with anti-p21, p53 and GAPDH antibodies. (right panel) Lysates from time = 0 were immunoblotted with anti-CBP, -p300, -p53, -GAPDH, and -p21 antibodies. (B) P21 mRNA induction in IR-treated p300/CBP deficient cells. IR treated cells from (A) were used to prepare mRNA which was then analyzed by p21 QRT-PCR.



Differential regulation of p53-dependent PUMA expression after DNA damage by p300 and CBP

Though neither p300 nor CBP were individually required for p21 induction after IR, their role in coactivating p53-dependent apoptotic target gene expression could not be evaluated after 10 Gy IR treatment of U2OS cells, as typical p53-dependent apoptotic target genes, such as PUMA, were not induced with this treatment (data not shown). To determine if p300 or CBP might coactivate, or otherwise regulate, the expression of a p53-activated apoptotic gene, control, p300-sh or CBP-shA cells were exposed to doxorubicin (Dox; 2 μM) and analyzed over a 24 hour time course for p53 (protein), PUMA and p21 (protein/mRNA) expression (Fig. 3.2A-B). Levels of p53, PUMA, p21 and HSP70 loading control from lysates of untreated (time = 0 hrs) cells were additionally analyzed on the same immunoblot (Fig. 3.2A, right panel) to allow comparison of protein abundances among the separate immunoblots that were needed to analyze the Dox treatment time course (Fig. 3.2A, left 3 panels).

p53 induction was overall similar among the 3 cell lines after Dox treatment (Fig. 3.2A). Of note, p21 protein and even mRNA levels were not substantially induced by Dox in any of the cell lines (Fig. 3.2 A,B). Since the analysis of p21 levels in control, CBP-shA and p300-sh cells treated with IR (10 Gy) (Fig. 3.1A-B), revealed the expected induction of p21. The p53-p21 signaling pathway is functioning properly in U2OS cells.

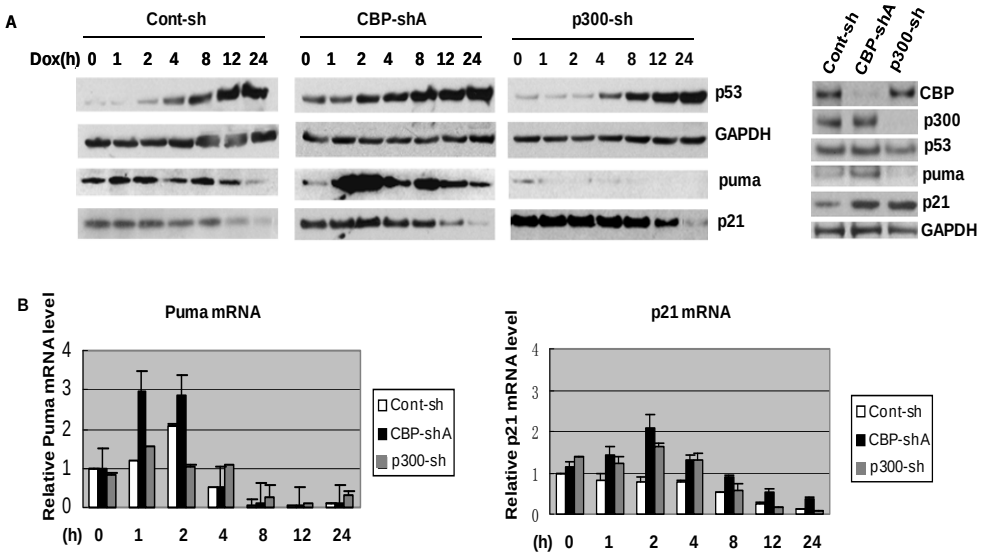
In contrast to p21, PUMA protein and mRNA were modestly induced by Dox in

control cells (Fig. 3.2 A, 1st panel; 3.2B). Unexpectedly, CBP loss caused an exaggerated induction of PUMA protein and mRNA (Fig. 3.2A, 2nd panel; Fig. 3.2B), while p300 deficiency caused near loss of PUMA protein signal (Fig. 3.2A, 3rd panel) and significant inhibition of PUMA mRNA induction, especially at 2 hrs (Fig. 3.2B). PUMA protein levels in p300-sh cells were not generally reflected in mRNA levels at most time points, except at the key 2hr time point where PUMA protein induction peaked in cont-sh and CBP-shA cells (Fig. 3.2A) and the PUMA mRNA level was coordinately 50% lower than the value in control cells (Fig. 3.2B).

Thus p300 and CBP appear to play opposing roles in the regulation of PUMA transcription after DNA damage. p300 action is consistent with its expected role as a coactivator, whereas CBP was a PUMA transcription repressor, where CBP loss led to derepression and higher basal and induced levels of PUMA mRNA and protein.

Fig. 3.2 Characterization of p53 target gene expression after Dox treatment of CBP and p300 stably-deficient cells.

Control, CBP-shA, and p300-sh U2OS cells were treated with Dox (2uM).Cells were collected at the indicated time points followed by determination of PUMA, p21, p53, and GAPDH protein abundance by immunoblot (A), and PUMA as well as p21 mRNA levels by QRT-PCR (B). The levels of CBP, p300, p53, p21, and PUMA at time=0 were determined on the same immunoblot to allow comparison among data from different immunoblots used for the time course analysis (A, right panel). Error bars indicate +/- 1 S.D.

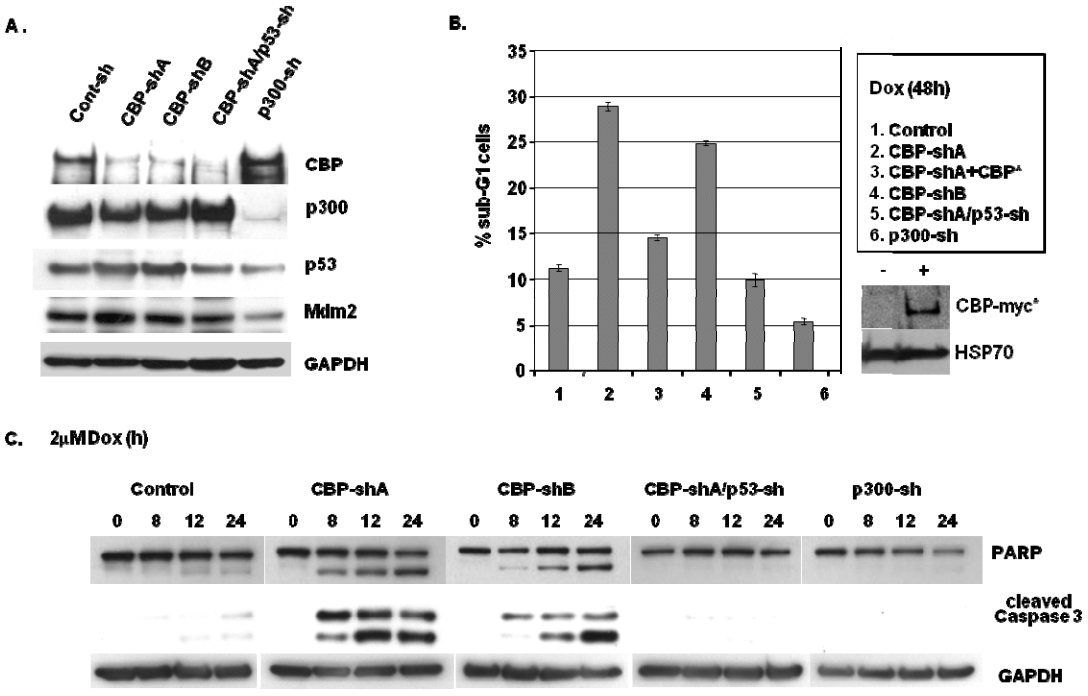


Differential regulation of apoptosis after DNA damage by p300 and CBP

To determine if the differential regulation of p53-dependent PUMA induction by p300 and CBP was of physiologic significance, p300 or CBP-deficient cells were treated with Dox, and cells were monitored for sub-G1 DNA content and cleavage of caspase 3 (Fig. 3.3). An increased sub-G1 fraction and induction of caspase 3/PARP cleavage were seen compared with control cells in cell lines expressing two distinct CBP shRNA's (CBP-shA and shB) after exposure to Dox, while p300-sh cells demonstrated cleaved caspase 3/PARP levels and sub-G1 fraction below that seen in controls (Fig. 3.3A-C). Rescue of the apoptotic phenotype of Dox-treated CBP-shA cells by expression of shRNA-resistant CBP cDNA (CBP*; the apoptotic fraction decreased from 28% to baseline levels) ruled out an off-target effect of the CBP shRNA (Fig. 3.3 B). Depletion of p53 in CBP-shA cells (partial p53 knock-down in CBP-shA cells results in reduction in p53 level to that observed in control cells) completely abrogated Dox-mediated accumulation of sub-G1 cells (Fig. 3.3A-C), consistent with a p53-dependent mechanism for apoptosis in CBP-shA cells after Dox treatment.

Fig 3.3 Analysis of p53 induced apoptosis in U2OS cells after CBP and p300 depletion.

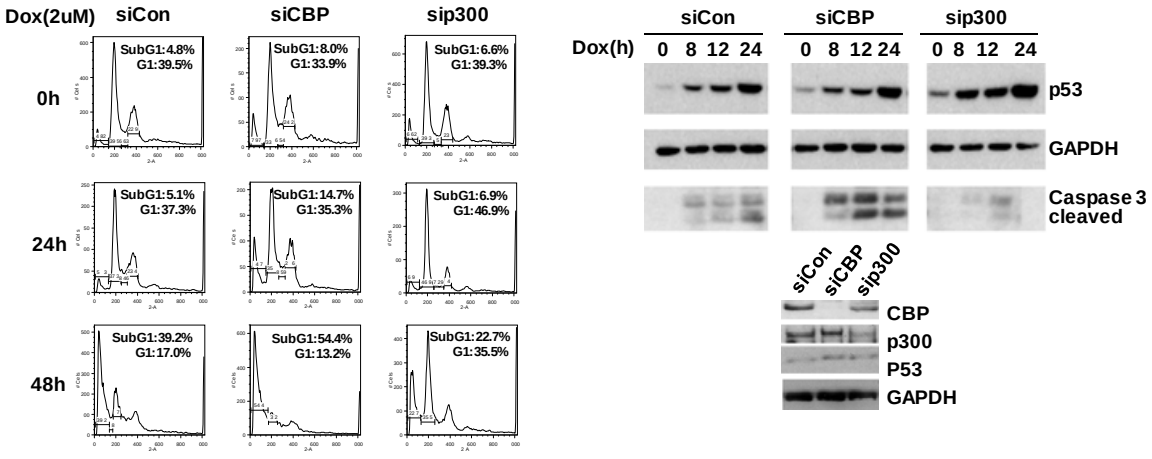
(A) The protein abundance in the indicated stably deficient U2OS cells. Lysates of drug-selected clones of U2OS cells harboring the indicated control (empty vector), CBP(A/B), p300 and CBP(A)/p53 shRNAs, were analyzed by immunoblotting with the indicated antibodies. (B) Differential regulation of apoptosis in p300 and CBP stably-deficient cells. Control, CBP-shA and B, p300-sh and CBP-shA/p53-sh U2OS cell lines with or without the indicated transfected (48 hr prior) CBP* rescue allele was exposed to Dox (1 μM) and then harvested at 48 hours for PI staining and FACS analysis for sub-G1 DNA content. Error bars indicate +/- 1 S.D. and are the average of 3 independent experiments. (C) Analysis of apoptotic markers in the indicated deficient U2OS cells. Lysates were prepared at the indicated times after Dox treatment for immunoblotting with anti-PARP, cleaved caspase 3, or -GAPDH antibodies. 1st/2nd panels and 3rd/4th panels were run on 2 separate gels. Gels were probed with antibody and exposed to film at the same time, allowing use of GAPDH to compare signal between gels.



To further confirm the specificity of these findings, and to control for the possibility of selection-based compensating mechanisms, U2OS cells were transiently depleted of p300 or CBP using siRNA, followed by Dox treatment and apoptotic analysis. Echoing the results with shRNA knockdowns of p300/CBP, CBP siRNA-treated cells exhibited substantially higher activated caspase 3 levels and sub-G1 apoptotic fraction at 48 hrs (54% vs. 39%) compared to control siRNA-treated cells, whereas p300 siRNA treatment resulted in a lower sub-G1 fraction (23% vs. 39%) and caspase 3 activation (Fig. 3.4). Of note, the G1 fraction was greatly enhanced after p300 silencing at 48 hrs after Dox, presumably as a result of the absence of apoptosis and redistribution of cells destined for apoptosis into the G1 fraction (36% vs. 6%; Fig. 3.4)

Fig. 3.4 Differential regulation of the cellular response to DNA damage in p300 and CBP siRNA treated cells.

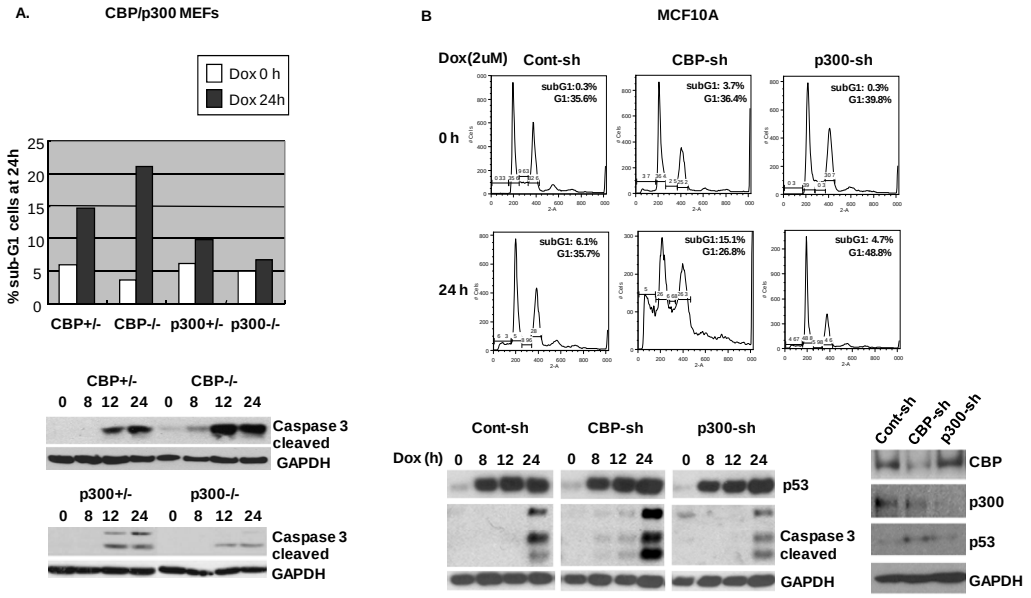
(left) U2OS cells transfected with control, CBP and p300 siRNA for 72 hours were treated with Dox (2 uM) and the cells were harvested at 0, 24, 48 hours after treatment for DNA content analysis by PI staining and FACS. (right) Analysis of p53 induction and apoptosis in U2OS cells after CBP and p300 depletion. (top) Lysates were prepared at the indicated times after Dox treatment for immunoblotting with anti-p53, anti-cleaved caspase 3, or GAPDH antibodies. (bottom) Lysates from time = 0 were immunoblotted with anti-CBP, p300, p53, GAPDH antibodies.



Ruling out cell/species specificity or transformation dependence of these observations, Dox-treated CBP-null MEFs exhibited higher, and p300-null MEFs lower, sub-G1 apoptotic fraction and caspase 3 activation, when compared with heterozygote littermate controls (Fig. 3.5 A). Likewise, non-transformed MCF10A cells exhibited moderately increased apoptosis when CBP was stably depleted (sub-G1 fraction 15% vs. 6% in control cells) (Fig. 3.5 B). As seen in the U2OS cells, p300 depletion caused an exaggerated G1 accumulation (49% G1 fraction in p300-depleted cells vs. 36% in control cells), though only a marginal reduction in apoptosis (5% sub-G1 fraction in p300 deficient cells vs. 6% in control cells) was seen after p300 silencing because the baseline level (in control cells treated with Dox) of apoptosis in these cells was low to begin with (Fig. 3.5B). Taken together, these results confirmed that p300 and CBP play separate, yet consistent, roles in directing p53 biologic responses towards arrest or apoptosis in diverse cell types and settings.

Fig. 3.5 DNA damage induced apoptosis in CBP or p300-deficient non-transformed cells.

(A) DNA damage induced apoptosis in CBP and p300-deficient MEFs. (top panel) CBP(+/-, -/-) and p300 (+/-,-/-) MEFs were treated with Dox (2 μM) and were collected at 0 and 24 hours for propidium iodide staining and FACS analysis for sub-G1 DNA content. (bottom panels) Lysates were collected from the cells treated with Dox at the indicated time points and immunoblotted with anti-cleaved caspase 3 and GAPDH antibodies. (B) CBP and p300 differentially regulate DNA-damage induced apoptosis in MCF10A cells. (top) Control, CBP, and p300 shRNA-expressing MCF10A cell lines were exposed to Dox (2 μM) and then harvested at 0 and 24 hours for propidium iodide staining and FACS analysis for sub-G1 DNA content. (bottom left) Lysates were collected from the cells treated with Dox at the indicated time points and were immunoblotted with anti-p53, cleaved caspase 3, and GAPDH antibodies. (bottom right) Lysates of cells at time 0 were immunoblotted with anti-p300, CBP, p53, and GAPDH antibodies.

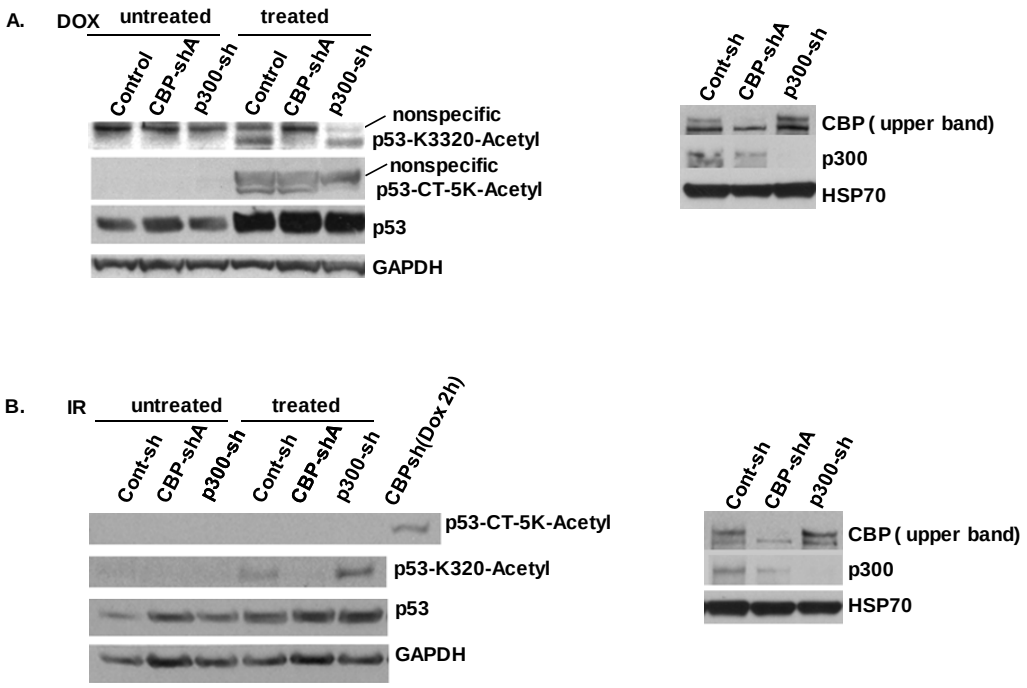


CBP and p300 differentially regulate C-terminal acetylation after DNA damage

Differential acetylation at p53 lysines 320 vs. 382 has been suggested to regulate the promoter specificity and downstream function of p53, such that lys-382 acetylation favors PUMA activation and apoptosis, whereas lys-320 acetylation favors p21 induction and G1 arrest ((83)). Thus, lys-320 and lys-370/372/373/381/382 (C-terminal cluster or "CTC") acetylation after Dox or IR treatment was evaluated in control, CBP-sh and p300-sh cells (Fig. 3.6). Though CBP depletion did not affect acetylation of the CTC, p300 loss substantially decreased the abundance of CTC-acetylated p53 after Dox (Fig. 3.6A), whereas IR did not induce CTC acetylation in any of the cell lines (Fig. 3.6B). This observation may correlate with the absence of apoptosis induced by IR in U2OS cells (data not shown). In contrast, CBP, but not p300 was required for Dox and IR-induced lys-320 acetylation (Fig. 3.6A-B). Therefore, CBP and p300 exhibit non-overlapping functions for stress-induced p53 acetylation that correlates with their apparent non-overlapping functions in the p53-dependent DNA damage response—p300 acetylates the CTC which is required for proper PUMA transactivation and apoptosis after DNA damage, while CBP participates, directly or indirectly, in K320 acetylation, which may negatively regulate PUMA and thus negatively impact the apoptotic response.

Fig. 3.6 Differential p53 acetylation pattern after DNA damage in CBP and p300 stably-deficient cells.

(left panels) Control, CBP-shA and p300-sh U2OS cells were treated with Dox (2uM, 6hrs) (A)or IR (10 Gy, 2hrs) (B) and lysates were immunoblotted with anti-p53 acetyl-K320, -p53 acetyl-CTC (lysines 370/372/373/381/382), -p53, -PUMA and -GAPDH antibodies. (right panels) Lysates (time=0) were immunoblotted with anti-CBP, -p300, and -hsp70 (loading control) antibodies.



CBP, but not P/CAF, is the major regulator of p53 lys-320 acetylation after DNA damage

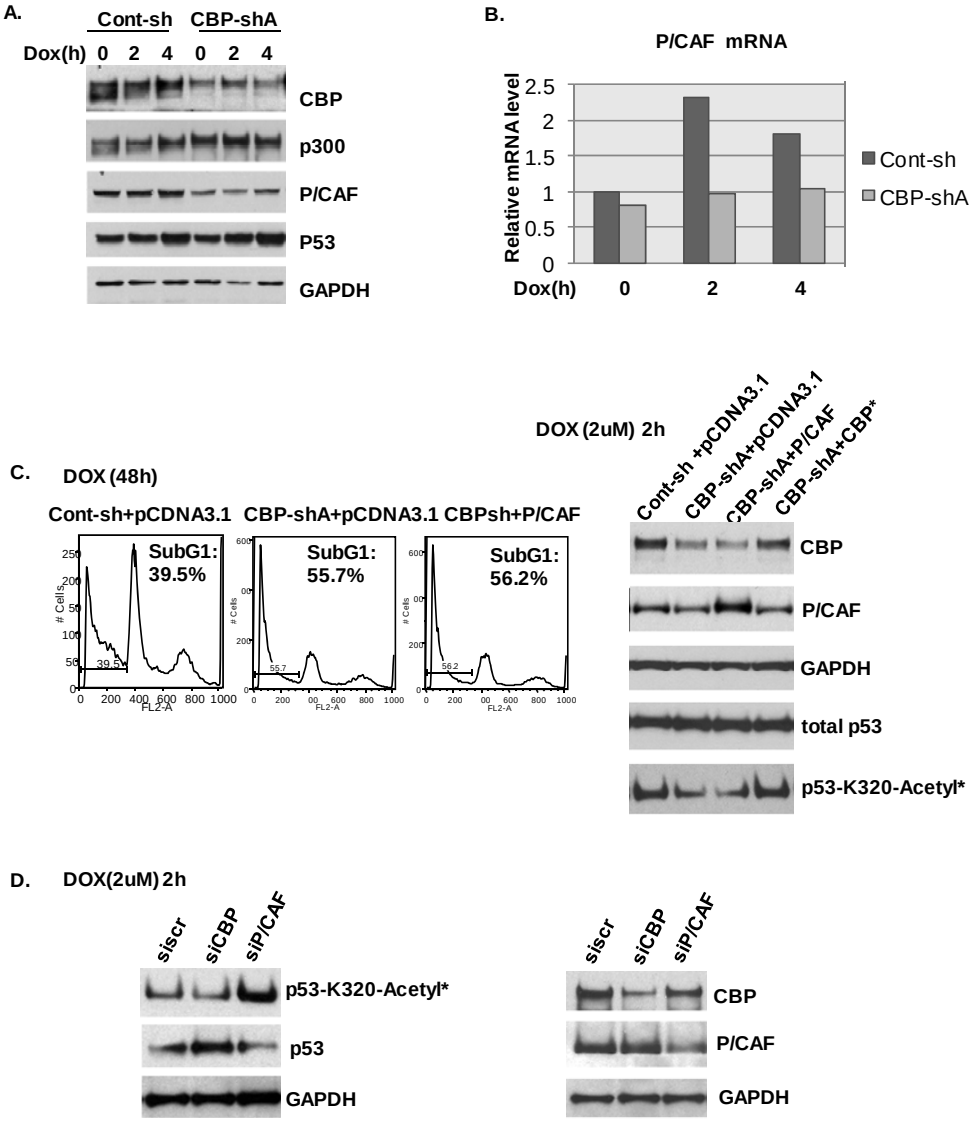
As P/CAF is the major lys-320 HAT, P/CAF abundance was measured in CBP deficient cells. Consistent with the loss of lys-320 acetylation in CBP-shA cells, P/CAF protein levels were markedly reduced, even after Dox treatment (Fig. 3.7A). The mechanism for the loss of P/CAF expression may be due, in part, to the reduced mRNA expression, which was most notable after Dox treatment, but did not explain the decreased protein abundance in untreated cells (Fig. 3.7B). P/CAF cDNA expression in CBP-shA cells was unable to rescue either apoptosis or lys-320 acetylation after Dox (Fig. 3.7C) suggesting that either it is not the lys-320 HAT, or that it must function in concert with another factor(s) to effect lys-320 acetylation. CBP would be the most likely candidate for a P/CAF cofactor, but CBP cDNA alone was sufficient to rescue the loss of lys-320 acetylation (Fig. 3.7C). Moreover, P/CAF siRNA knockdown did not negatively impact Dox-induced K320 acetylation, and surprisingly K320 acetylation was further induced in cells exposed to Dox and P/CAF siRNA (Fig. 3.7D).

Therefore, CBP and p300 exhibit non-overlapping functions for stress-induced p53 acetylation that correlates with their apparent non-overlapping functions in the p53-dependent DNA damage response—p300 acetylates the CTC which is required for proper PUMA transactivation and apoptosis after DNA damage, while CBP, but not

P/CAF, participates, directly or indirectly, in K320 acetylation, which may negatively regulate PUMA and thus negatively impact the apoptotic response.

Fig. 3.7 CBP, but not P/CAF, is the major regulator of p53 lys-320 acetylation after DNA damage.

(A,B) Characterization of P/CAF gene expression after Dox treatment of CBP stably-deficient cells. Control and CBP-shA U2OS cells were treated with Dox (2 uM). Cells were collected at the indicated time points followed by determination of P/CAF, CBP, p53, and GAPDH protein abundance by immunoblot (A), and P/CAF mRNA levels by QRT-PCR (B). (C) (left) Control and CBPshA U2OS cells transfected with the indicated (48 hr prior) P/CAF or pCDNA3.1 alleles were exposed to Dox (2 μM) and then harvested at 48 hours for PI staining and FACS analysis for sub-G1 DNA content. (right) Control and CBPshA U2OS cells transfected with the indicated transfected (48 hr prior) P/CAF, CBP or pCDNA3.1 alleles were treated with Dox (2 uM) for 2 hrs and lysates immunoblotted with anti-p53 acetyl-K320, P/CAF, CBP and GAPDH antibodies. (D) U2OS cells transfected with control, CBP and P/CAF siRNA for 72 hours were treated with Dox (2 uM) and the cells were harvested at 0, 2 hours after treatment for immunoblot of anti-CBP, P/CAF and GAPDH antibodies at 0 hour and the anti-p53, p53 acetyl-K320, GAPDH antibodies at the 2 hours. p53-k320-Acetyl* indicates the signal is obtained by p53-K320 immonoprecipitation followed by p53 immunoblotting.


A.
Cont-sh
CBP-shA
Dox(h) 0 2 4 0 2 4
CBP
p300
P/CAF
P53
GAPDH
B.
P/CAF mRNA
Relative mRNA level
2.5
2
1.5
1
0.5
0
Dox(h) 0 2 4
Cont-sh
CBP-shA
C.
DOX (48h)
Cont-sh+pCDNA3.1
CBP-shA+pCDNA3.1
CBPsh+P/CAF
SubG1: 39.5%
SubG1: 55.7%
SubG1: 56.2%
Cells
FL2-A
DOX (2uM) 2h
Cont-sh +pCDNA3.1
CBP-shA+pCDNA3.1
CBP-shA+P/CAF
CBP-shA+CBP*
CBP
P/CAF
GAPDH
total p53
p53-K320-Acetyl*
D.
DOX(2uM) 2h
siscr
siCBP
siP/CAF
p53-K320-Acetyl*
p53
GAPDH
siscr
siCBP
siP/CAF
CBP
P/CAF
GAPDH

3.4 Discussion

p300 and its paralog CBP have been identified as physiological regulators of p53 ubiquitination and stability. The hyperactivation of PUMA in CBP-shA cells and p21 in p300-sh and CBP-shA cells suggested that these p53 target genes may be under paradoxical negative regulation by p300/CBP. Surprisingly, neither CBP nor p300, on their own, were required for Dox (or IR)-induced transcription of p21, but p300 was required for physiologic induction of PUMA transcription. Moreover, CBP negatively regulated PUMA after Dox treatment, while p300 and CBP both contributed to the negative regulation of p21 transcription in unstressed and Dox or IR-treated cells. Differential transcription regulation by p300/CBP were correlated with differential effects on p53 acetylation, with K320 acetylation, a negative regulator of apoptotic gene expression (18), dependent on CBP, while CTC acetylation, a positive regulator of apoptotic gene expression (83), was dependent only on p300.

Individual loss of CBP or p300 did not reveal a defect in coactivation of p53 gene targets. This may be due to complete redundancy for this function. Instead, CBP loss, alone, led to a gain in p53 activation of PUMA expression, while increased p21 expression was seen after p300 or CBP loss (Fig. 3.1 and Fig. 3.2). The increase in p21 level after p300 or CBP depletion cannot be explained by specific defects in acetylation, but likely reflect some alteration of p53-chromatin dynamics that is regulated in a common fashion by p300 and CBP. In contrast, K320 acetylation,

which is defective only in CBP-deficient cells, has been reported as a negative regulator of apoptosis correlated with decreased pro-apoptotic gene expression (18, 83).

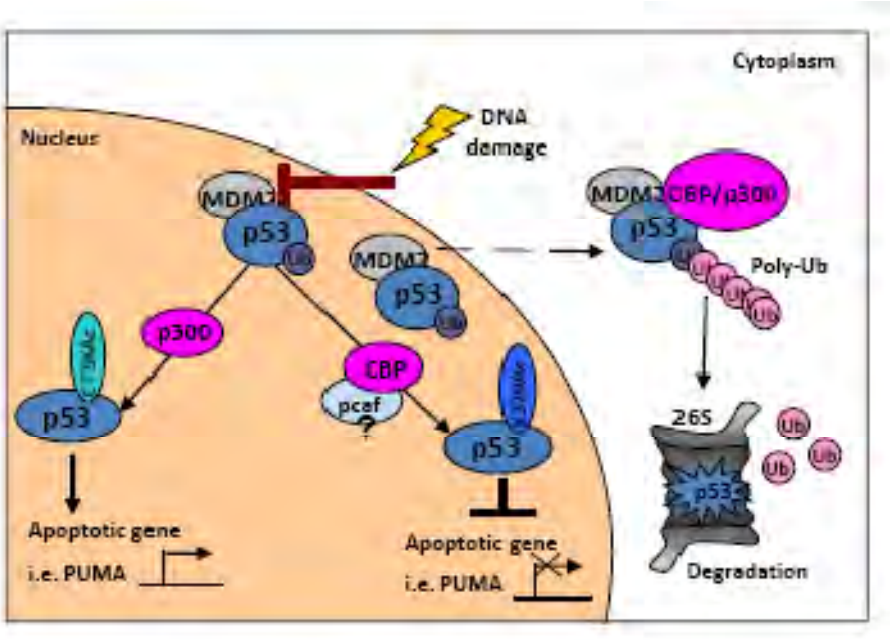
The mechanism by which p53 acetylation might negatively regulate p53 transactivation at specific genes is unknown and may include either or both recruitment of repressor complexes or the inability to recruit activating transcription factors. Indeed, CBP can be found in complexes with the SWI/SNF factor BRG1 (61), and a BRG1/CBP complex may act to repress p53 target genes, preventing their activation under normal (unstressed) proliferative conditions (113). Additionally, acetylation is known to affect promoter-binding preference by p53, and this mechanism may also contribute to the observed effect of CBP deficiency and decreased K320 acetylation in increasing PUMA expression (83). The loss of CTC acetylation in p300-deficient cells correlated with lower PUMA levels, suggesting a loss of coactivation. This effect was reflected partly at the mRNA level with a loss of DNA damage induction of PUMA expression, combined with other unknown post-transcriptional effects of p300 on PUMA expression that led to the much lower basal protein levels as well. The mechanism of CTC regulation of PUMA expression may likewise be operative at the level of coactivator recruitment or DNA binding preference, or as has been recently suggested, by destabilization of MDM2/p53 interaction at target promoters, releasing MDM2 inhibition (141).

The mechanism for differential acetylation of p53 by p300/CBP is unclear. Multiple interacting factors influence p300/CBP acetylation of p53, including STRAP (28), and ING2 (120). Of these, ING2 is intriguing as it appears to specifically stabilize K382 acetylation by p300 (120), raising the question of whether another related ING protein (136) might similarly influence K320 acetylation. If so, perhaps these factors also exhibit specificity for interacting with either p300 or CBP.

As a factor that normally maintains p53 instability and suppresses apoptosis, CBP would be predicted to be maintained in tumors, and thus also represents a potential therapeutic target for activating p53. p300, conversely, is mutated, albeit at low frequency, in certain human malignancies (50, 70) possibly due to the negative impact on the p53 pathway, and specifically p53-induced apoptosis. Given that cancer cells harboring wild-type p53 are specifically primed to undergo apoptosis when p53 is activated (14), mechanisms such as specific CBP inhibition may be of great value in cancer therapy, by increasing the therapeutic index of highly toxic chemotherapeutics such as doxorubicin.

Fig. 3.8 Distinct p53 acetylation patterns control cell fate.

CBP and p300 regulate p53 ubiquitination and acetylation patterns in a compartment-specific fashion, maintaining p53 instability in the absence of stress in cytoplasm, and selectively regulating transcription of distinct p53 downstream targets to control cell fate after a stress such as DNA damage in nucleus, by modulating p53 acetylation patterns. p300 –dependent CTC acetylation correlates with robust PUMA induction, while CBP-dependent (direct or indirect) K320 acetylation correlates with repression of PUMA.



3.5 Materials and Methods

Cell culture and plasmids.

U2OS cells were grown in DMEM medium supplemented with 10% FBS and antibiotics. CBP (+/-, -/-) or p300 (+/-, -/-) mouse embryonic fibroblasts (36, 155); Kung et al. 2000) were maintained in DMEM/10% FBS supplemented with 200 ug/ml G418. Cells were treated, where noted, with 100 μg/ml cycloheximide, 1-2 μM doxorubicin (Sigma), or 10 Gy IR. Plasmid transfection was done with Fugene 6 (Roche), and siRNA transfections employed Oligofectamine (Invitrogen). pRSV-CBPmyc (Kazantsev et al. 1999), pcDNA-UbHA wt, K48O, and K63O (78), and pCMVbp300CHA (Eckner et al. 1994) have been described. An shRNA-resistant CBP allele (pRSV-CBP*myc) was generated by silent mutation of the shRNA target sequence (AACTCCAATAGC mutated to AATAGTAACTCT; CBP residues 190-193) within pRSV-CBPmyc.

Generation of knockdown cell lines

Stable hairpin RNA (shRNA)-expressing cell lines were generated using the following shRNA target sequences: CATAAACAACTGTCGGAGC (CBP-shA), TAGTAACTCTGGCCATAGC (CBP-shB), GACTCCAGTGGTAATCTAC (p53-sh) and TCATTTCACACTGGAAGAA (p300-sh). The hairpin oligonucleotides were cloned into pSuperior.puro (Oligoengine) (p300 and CBP) or pTER+zeo (p53) using BglII/XhoI cloning sites. After transfection of the hairpin constructs, the cells were

subjected to selection with 1 μg/ml puromycin (AG Scientific) and for CBP-sh/p53-sh cells, 400μg/ml Zeocin was also added (Invitrogen). Independent clones were selected and evaluated for silencing by immunoblot.

siRNA transfection:

siRNA duplex corresponding to

CBP (forward:5'-AAUCCACAGUACCGAGAAAUGUU-3';

reverse: 5'-CAUUUCUCGGUACUGUGGAUUUU-3'),

p300 (forward:5'- CAGCGCAGUCCUGGACCAGtt-3';

reverse:5'-CUAAUCCAGGACUGCUCUGtt -3') and

Control (siGFP) were synthesized by Dharmacon. 1x10 7 U2OS cells were transfected with 0.2 pmol siRNA using Oligofectamine (Invitrogen). 72 hours after transfection, cells were harvested and analyzed for the expression level of CBP, p300, GAPDH, caspase 3 and p53 by western blotting, or were treated with doxorubicin as indicated.

Western blotting and immunoprecipitation.

For western blot analyses, cells were lysed in cold NETN240 buffer (20mM Hepes pH7.4, 2 mM $MgCl_2$, 10 uM $ZnCl_2$, 240 mM NaCl, 0.2% Triton X-100), supplemented with Complete EDTA-free protease inhibitor tablets (Roche). For p53

acetylation analysis, cells were treated with 1 mM trichostatin A (TSA) and 5 mM nicotinamide for 6 hours, then lysed in FLAG lysis buffer (50 mM Tris•HCl, pH 7.8, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 1 mM DTT, 10% glycerol, 10 mM trichostatin A (TSA) and 5 mM nicotinamide and fresh protease inhibitors). For immunoprecipitation, cells were lysed in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, 10μM $ZnCl_2$, 1% Triton X-100, 0.5% DOC supplemented with fresh 5mM NEM and Complete EDTA-free tablets (Roche)). Immunoprecipitations were performed in the lysis buffer overnight, followed by capture with Protein A agarose (Upstate) and five washes in lysis buffer. Antibodies used for immunoblotting were: αp53-DO1, αp53-FL393R , αUb P4D1, αCBP A22, αHSP70 W27, αp21 C19, αMdm2 N-20 (all Santa Cruz), αPUMA C-T (Sigma), αGAPDH 6C5 (Advanced Immunochemical), αmyc-tag 4A6, αp300 RW128 (Upstate) and αRb (BD Pharmingen), αp53-Acetyl K320 (Upstate), αp53-Acetyl C-terminal 5K(370/372/373/381/382) (Gu and Roeder, 1997) αphospho-p53 (Ser15) (Cell Signaling) . Western blot signals were quantified after visualization of primary antibody by HRP-conjugated secondary antibody and enhanced chemiluminescence or by fluorescent-labeled secondary antibody and detection by Odyssey blot scanner (LiCor), using ImageJ NIH software.

Quantitative Real-time PCR: Total RNAs were extracted from U2OS cells at the indicated time points with RNeasy (Qiagen). cDNA synthesis was performed with AffinityScript (Stratagene), using 1 μg of total RNA. Quantitative RT-PCR was

performed using Power SYBR Green (Applied Biosystems). The following primers were used:

p53: F5’-GCGTGAGCGCTTCGAGAT-3’,

R5’-AGCCTGGGCATCCTTGAGT-3’

PUMA: F5’-GGGCCCAGACTGTGAATCC-3’,

R5’-CGTCGCTCTCTCTAAACCTATGC-3’

p21:F5’-TGGAGACTCTCAGGGTCGAAA-3’,

R5’-GCGTTTGGAGTGGTAGAAATCTG-3’

GAPDH: F5’-TGTTCGACAGTCAGCCGC-3’,

R5’-GGTGTCTGAGCGATGTGGC-3’

Chapter 4 S5a is a negative regulator of p53 stability and activity.

4.1 Abstract

The pathway of p53 degradation is a complicated, multi-step procedure, which includes monoubiquitination, polyubiquitination and proteasome delivery. This study focuses on the proteasome delivery step in the p53 degradation pathway. We discovered that S5a, a 19S proteasome subunit, might be the entry point for hHR23 to the 26S proteasome to mediate p53 degradation. S5a was shown to physically interact with hHR23 in U2OS cells, and S5a was limiting for p53 degradation. S5a depletion caused accumulation of both native and oligoubiquitinated p53 due to impaired proteasome degradation. S5a was also characterized as a negative regulator of p53 transactivation possibly through its presence at p53 target gene promoters. The role of S5a in both degradation and transcription regulation of p53 indicates that the p53 ubiquitination/degradation and transcriptional machinery might function coordinately.

4.2 Introduction

p53 activity and function are tightly controlled by the ubiquitin/proteasome degradation system (UPS). p53 protein is rapidly degraded in normal cells due to its constitutive ubiquitination in the absence of stress, but it becomes quickly stabilized upon cellular stress, due to the effective inhibition of specific p53 ubiquitination, proteasome degradation or both. A few E3 and E4 ubiquitin ligases, have been reported to regulate p53 polyubiquitination, including MDM2, Arf-BP1, E6-AP, COP1, Pirh2 and p300/CBP. Beyond E3/E4 activities, additional steps appear to regulate p53 turnover prior to its final degradation by the 26S proteasome.

HHR23 (yeast Rad23) is a UBL-UBA protein which encodes an NH_2-terminal conserved UBL domain that interacts with the 26S proteasome, and tandem UBA domains which specifically bind to K48-linked polyubiquitin chains. This structure of UBL-UBA proteins suggests that they may serve as scaffolds or adaptors for the concerted ubiquitination and proteasome degradation of substrates. In support of this hypothesis, overxpression or siRNA depletion of hHR23 influences p53 stability (10). In addition, overexpression of a dominant negative hPLIC1 (yeast Dsk2), another UBL-UBA protein, stabilizes p53 (81). Based on their structures and effects on p53 when depleted or overexpressed, hHR23 and hPLIC1 may deliver polyubiquitinated p53 to the proteasome. Finding out the entry point of hHR23 on the proteasome would therefore be an interesting subject for understanding both the general

mechanism of substrate delivery to the proteasome, as well as the specific mechanism of p53 degradation by the proteasome.

The S5a subunit of the 19S regulatory particle has been characterized as the receptor for a subset of proteasome substrates (107). Some evidence points to its possible role as a ubiquitin receptor able to bind ubiquitinated substrates directly via one or both (in human) of its ubiquitin interaction domains (UIMs) (39) . More recent evidence, however, has suggested that the UIM domains of S5a exhibit a binding preference for UbL domains over native ubiquitin (137). Therefore, S5a is proposed to capture substrates using hHR23, or other UBL-UBA proteins, which can bridge ubiquitinated substrates to the proteasome through their UBA domains (21). In yeast, however, only a specific subset of proteasome substrates require Rad23 and/or S5a for degradation, including sic1 and far1 (23), suggesting multiple parallel routes for substrate recognition and entry to the proteasome. Taken together, the available data in the literature suggests that hHR23, and/or a related UBL-UBA protein, may bring polyubiquitinated p53 to S5a, leading to proteasome degradation. Based on this hypothesis, we initiated a comprehensive study of S5a function in p53 regulation to fully understand its mechanism of degradation downstream of the polyubiquitination steps.

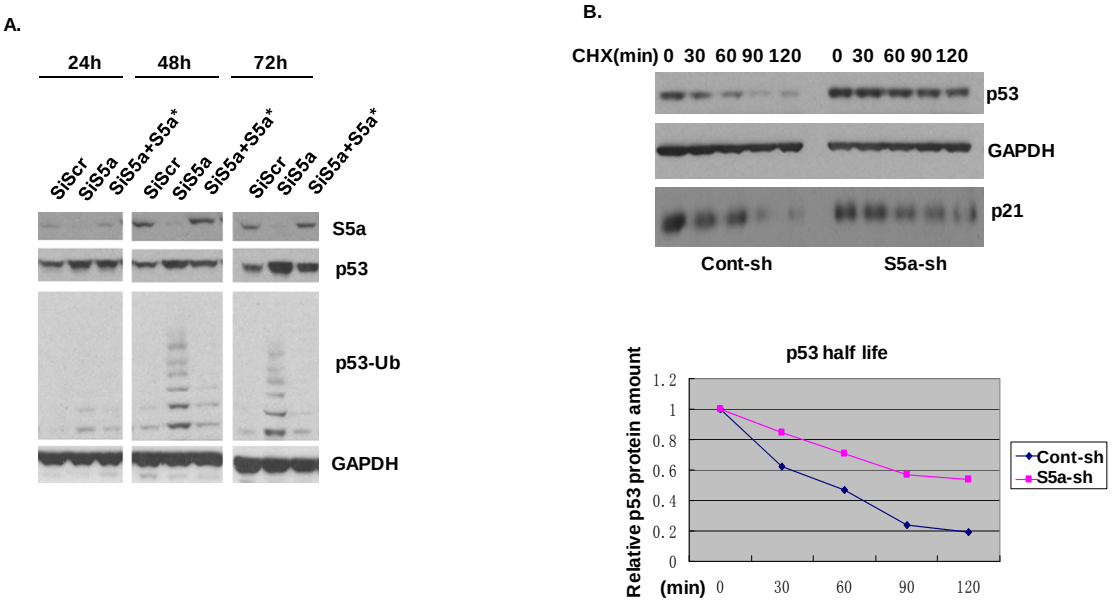
4.3 Result

S5a is required for p53 degradation in unstressed cells.

To determine the role of S5a in p53 homeostasis, p53 levels were analyzed in U2OS cells treated with control or S5a siRNA. Both native and ubiquitin conjugated p53 levels were elevated after S5a depletion (Fig.4.1A). The phenotype was completely reversed by rescue expression of siRNA-resistant S5a cDNA in si-S5a treated cells, ruling out an off-target effect of the siRNA (Fig. 4.1A). Similar increases in p53 abundance were seen in U2OS cells expressing 2 independent S5a shRNAs vs. control shRNA (data not shown). Moreover, cycloheximide decay analysis of control and an S5a-sh cell line, revealed that p53 half-life was prolonged (>2 hr vs. 1 hr) in the absence of S5a (Fig. 4.1 B). S5a is a receptor for many, but not all, proteasome substrates, and other known and unknown subunits serve as proteasome substrate gateways (147) . These data suggest that p53 is targeted to the proteasome primarily via S5a.

Fig. 4.1 S5a is required for physiologic p53 turnover in unstressed cells.

(A) U2OS cells were treated with control or S5a siRNA for 72 hours. S5a siRNA-treated cells were also transfected with S5a expression vector (siRNA-resistant; S5a*) 48 hrs before harvest. Lysates were immunoblotted with anti-S5a, -p53 and –GAPDH antibodies. p53-Ub indicates longer exposure blot to demonstrate accumulation of oligoubiquitinated p53 in siS5a-treated cells. (B) Cycloheximide-decay analysis of control and S5a shRNA expressing cell lines. p53 half-life increased from 60 min in control cells to >120 min in S5a-sh cells.

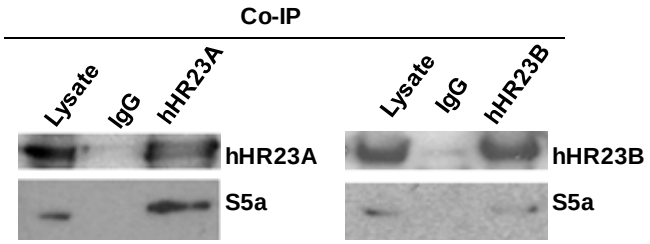


S5a interacts with hHR23 in vivo

Though S5a may capture ubiquitinated substrates directly, or indirectly via UBL-UBA adaptor proteins (147), UBL domains appear to be its preferred binding partner (112). HHR23/S5a interaction is well characterized in vitro (124). To confirm that S5a and hHR23 (hHR23A and hHR23B are human paralogs) interact in vivo, U2OS cell lysates were immunoprecipitated with IgG, anti-hHR23A or anti-hHR23 B antibodies, and the IP's immunoblotted for hHR23A or B and S5a (Fig. 4.2). The control IgG IP's demonstrated no detectable S5a, indicating very low background binding of S5a to the IP antibody or protein A Sepharose. As predicted from the in vitro data, both the hHR23A and hHR23B IP's contained significant quantities of S5a, comparable to the amount seen in the lysate lanes, indicating that at least 10% of S5a was found in complex with either hHR23 paralog.

Fig. 4.2. S5a interacts with hHR23 in vivo.

U2OS cell lysates were IP'd with IgG, hHR23A (3), and hHR23B (BD) antibodies, and the IP's immunoblotted for hHR23A, hHR23B and S5a as indicated. Lysate indicates 10% of amount used in the associated IP.



S5a antagonizes p53 dependant apoptosis

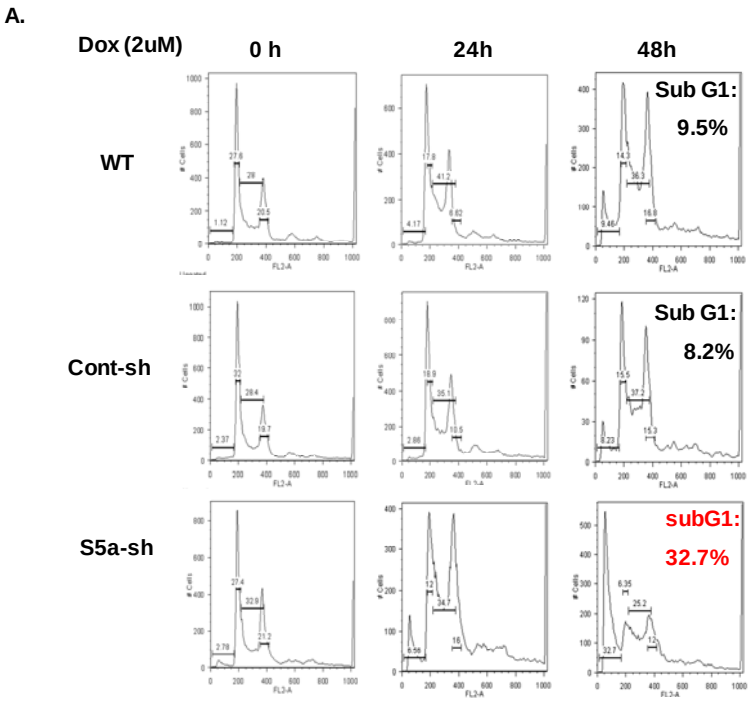
To understand the contribution of S5a to the p53 DNA damage response, normal U2OS, S5a-sh, or control-sh cell lines were treated with 2 uM Dox and assayed for the induction of apoptosis at 24 and 48 hrs. Depletion of S5a sensitized cells to Dox-induced apoptosis (at 48 hrs, % sub-G1 fraction= 9% in U2OS cells; 8% in control-sh cells; 33% in S5a-sh cells) (Fig. 4.3A). This increased apoptosis was consistent with the observed increase in p53 levels in the stably S5a-deficient cells (see Fig. 4.1A and 4.3A). The further depletion of p53 in S5a-sh cells abrogated Dox-mediated accumulation of sub-G1 cells (Fig. 4.3.B), indicating a p53-dependent mechanism for Dox induced apoptosis in S5a-shA cells.

Previous reports have suggested that elevated levels of p53 seen after proteasome inhibition are not necessarily correlated with elevated p53 functional activity (82). Therefore, to explain the increased apoptosis in S5a-sh cells, control-sh, S5a-sh and S5a-sh/p53-sh cells were treated with 2 uM Dox and assayed for p53 and p53 target gene (PUMA/p21) induction. Analysis of p53 abundance after Dox in S5a-sh cells demonstrated the expected modestly increased p53 levels over control cells (Fig. 4.3 C). The increased level of apoptosis in S5a-sh cells also correlated with a substantial increase in pro-apoptotic PUMA protein levels in both untreated and Dox-treated cells compared with control-sh cells (Fig.4.3 C). p21 levels were increased as

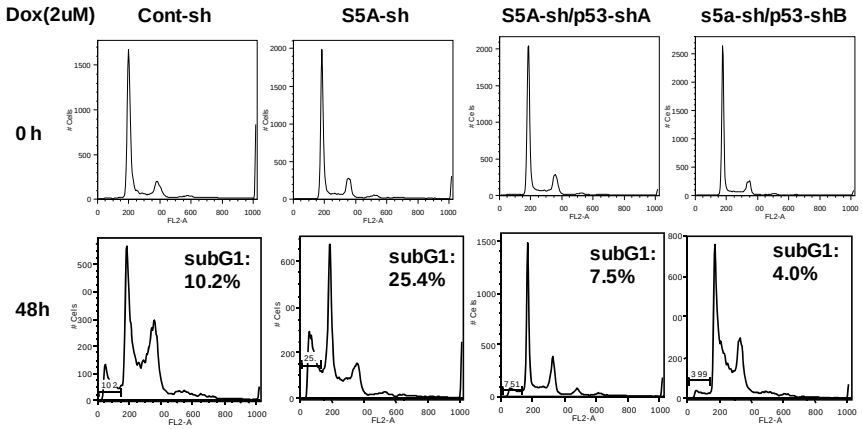
well. The increase in PUMA was dependent on p53 as depletion of p53 with shRNA abrogated the increase in PUMA in untreated and Dox-treated S5a-sh cells (Fig. 4.3 C).

Fig. 4.3 S5a antagonizes p53-induced apoptosis

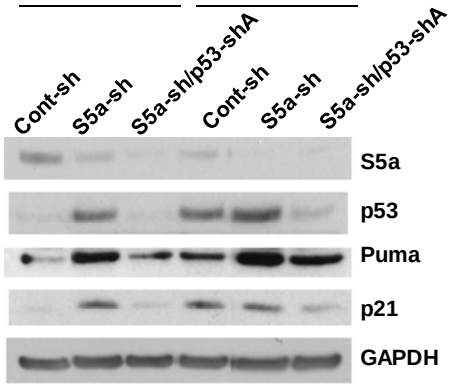
(A)Wild type, cont-sh, and S5a-sh U2OS cells were treated with Dox (2 uM) and the cells were harvested at 0, 24, 48 hours after treatment for DNA content analysis by PI staining and FACS. (B) Cont-sh, S5a-sh, and S5a-sh/p53-sh U2OS cells were treated with Dox (2uM) and the cells were harvested at 0 and 48 hours after treatment for DNA content analysis by PI staining and FACS. For S5a-sh/p53-sh cells, two clones, S5a-sh/p53-shA and S5a-sh/p53-shB, were analyzed for FACS. (C) Control, S5a or S5a/p53 shRNA-expressing cell lines were exposed to mock or Dox (2μM) treatment for 4 hrs. Lysates were immunoblotted for S5a, p53, PUMA, p21, and GAPDH.



B.



C. Dox(2uM) untreated treated

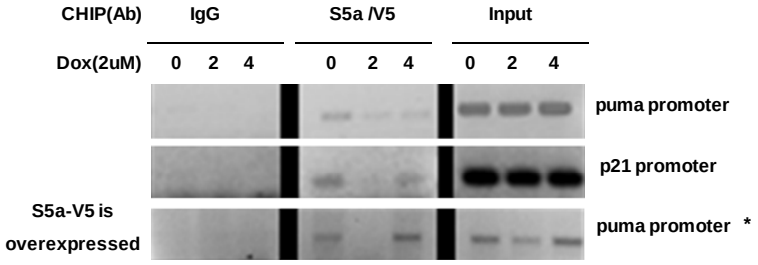


S5a associates with p53 target promoters

It has been proposed that the proteasome participates directly in transcription regulation (7, 44, 45). To determine if S5a localizes to p53 responsive promoters before or after genotoxic stress, U2OS cells were treated with Dox and cells were harvested for crosslinked chromatin preparation at 0, 2 and 4 hours after Dox treatment. The chromatin was subjected to S5a chromatin IP (ChIP), followed by p21 or PUMA promoter specific PCR for promoter fragments enriched in p53 binding sites (75). Consistent with a negative regulatory role for S5a in p53 transcription regulation, S5a was found coordinated to the promoter pre-stress, but was lost from the promoter at 2 hours post-treatment with Dox, followed by its binding to the promoters at 4 hours post-stress (Fig. 4.4). To better confirm the localization of S5a to the PUMA promoter, U2OS cells were also transfected with V5-S5a cDNA, followed by Dox treatment, S5a ChIP, and PUMA promoter PCR of the ChIP (Fig. 4.4, bottom panel). As seen with untransfected U2OS, but with a more robust signal, V5-S5a was present at the PUMA promoter pre-stress, lost from the PUMA promoter at 2 hr, and returned at 4 hr. Thus, the release of S5a from the PUMA and p21 promoters after DNA damage provides a mechanism by which p53-dependant transcription can be kept inhibited in resting cells, but rapidly activated after stress induction.

Fig. 4.4 S5a localizes to p53 target promoters

IgG, S5a or V5 ChIP of chromatin prepared from U2OS cells treated with Dox at the indicated times and analyzed with p21 and PUMA promoter PCR primers. To further amplify the PCR signal of the PUMA promoter, the V5-S5a cDNA expressing U2OS cells were processed for V5 CHIP and PCR with PUMA promoter primers.



4.4 Discussion

This study, even though very preliminary, suggests that p53 is targeted to the proteasome primarily via S5a. S5a depletion results in accumulation of both native and oligoubiquitinated p53 due to impaired proteasome degradation. S5a might also capture ubiquitinated substrates directly, or via UBL-UBA adaptor proteins, like hHR23. We have confirmed the physiological interaction between S5a and hHR23 in support of the model that hHR23 and S5a work sequentially to regulate p53 degradation.

S5a contributes not only to p53 degradation, but also to the p53 DNA damage response. Depletion of S5a sensitized cells to Dox-induced apoptosis. Though this phenotype seems consistent with the modest increase in p53 protein level in the S5a knockdown cells, we propose that S5a directly regulates p53 transcription activity based on our observation that S5a associates with the PUMA and p21 promoters in the vicinity of p53 binding sites.

There are at least two conflicting models for the role of the UPS in activated transcription, as would be the case for p53. Work with the Gal4 system clearly shows that the proteasome plays a negative role, constitutively stripping off DNA-bound transactivator by interacting with the activation domain, and subsequently unfolding the rest of the transactivator through a mechanism similar to the ATPase-mediated unfolding of proteasome substrates destined for degradation. The transcription

regulatory activity of the proteasome is non-proteolytic, requiring ATP but not blocked by proteasome protease inhibitors (45) . Alternatively, ubiquitination and degradation of activator/coactivator complexes have been proposed as a way of clearing initiation complexes to allow the loading of new initiation complexes to maintain active transcription (100). In this case proteolytic activity is required and proteasome inhibitors inhibit activation. Some reports do suggest that p53 activation is negatively affected by proteasome inhibition, but interpretation of these experiments is clouded by the nucleolar relocalization of p53 observed after proteasome inhibition (82, 123). The dynamics of S5a protein on the PUMA and p21 promoters in U2OS cells can be best correlated with a negative role for S5a, and very possibly, the 19S or 26S proteasome, in the regulation of p53 transactivation. We will get a better understanding of the role of the UPS in p53 transactivation with a more extensive analysis of S5a and other proteasome subunit association with p53 responsive promoters under a variety of stress conditions.

Overall, S5a negatively regulates p53 stability and transcriptional activity. The inhibition of S5a leads to increased p53 protein level, transactivation, and p53-induced apoptosis after DNA damage. Proteasome inhibitors specifically targeting S5a might be able to rescue p53 function in cancer cells, and therefore might serve as effective anti-cancer therapeutics alone or in combination with conventional genotoxics. S5a inhibitors might also cause less adverse effects when compared to the traditional therapeutic proteasome inhibitors, e.g. bortezomib. Since 1st generation proteasome

inhibitors target the 20S catalytic core of the 26S proteasome, all protein substrates theoretically accumulate, and subsequently their functions will be dysregulated. The S5a inhibitors will, by definition, exhibit relative selectivity, as they are predicted to target only a subset, but not all, protein substrates destined for proteasome degradation.

4.5 Materials and Methods

S5a shRNA sequence: CCAGCCAAGGAGGAGGATGATTA

S5a siRNA sequence : ACTGGCTAATGACTGTGAA

Chromatin immonoprecipitation assay (CHIP)

Cells were plated 24 hours before experiment. Cells from two 150mm culture dishes with 70-80% confluence were used for each CHIP sample. Cells were applied to washing with cold PBS two times and crosslinked with 1% formaldehyde for 10 mins at R.T., followed by the incubation with 125 mM (pH 3.0) glycine for 5 mins at R.T. to quench the formaldehyde. Cells then were washed twice with cold PBS. Nuclei were extracted by incubating with Nuclei Isolation Buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40) for 30 min on ice and subsequent centrifugation at 7,000g for 5 min in cold room. The nuclei pellets were lysed by RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA). After 30 mins incubation, the chromatin was sheared into 100-1000 bp long fragments by sonication. Nuclear debris was removed by centrifugation at 14000 rpm for 20 min. The supernatants were processed for immonoprecipitation with respective antibodies overnight in cold room. Protein A Sepharose beads were added into the complex and incubated for additional two hours in cold room. The beads then were washed sequentially in RIPA buffer, RIPA (500 mM NaCl) buffer, LiCl buffer (10 mM Tris HCl pH8.0, 250 mM LiCl, 0.5% NP40, 0.5% sodium deoxycholate , 1 mM EDTA)

and Tris-EDTA pH 8.0. The beads were then resuspended in 250 ul elution buffer (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 1% SDS) with proteinase K and incubated at 55 degree overnight. The supernatants were processed for DNA purification with phenol-chloroform method. In the final step, DNA was precipitated in the glycogen with ethanol and air dried. 50 ul Tris-EDTA pH 8.0 buffer was used to resolve each sample and 5ul of immonoprecipitated DNA was used in PCR reaction for each promoter region.

Chapter5 Conclusions and future perspectives

The p53 tumor suppressor responds to a wide range of stresses, including chemical mutagens, ionizing radiation, hypoxia, and nutrient deprivation, by activating growth arrest or apoptosis programs. Both apoptosis and cell cycle arrest functions have been confirmed in attribution to its tumor suppressor function in animal models. All cancers must inactivate the p53 network at one or more of its nodes to progress. Most often, p53 is mutated such that it is inactivated in tumors. In other cases, regulators of p53 are altered instead of p53 itself. Frequently, the regulators that are altered in cancer cells participate in the normal destruction of the p53 protein by the ubiquitin and proteasome system (UPS). For example, MDM2, E3 ubiquitin ligase of p53, is often amplified in the tumor cells containing wild type p53; Arf, an inhibitor of MDM2, is commonly silenced in cancer cells.

The pathway of p53 destruction by the ubiquitin and proteasome system is complex, involving monoubiquitination, polyubiquitination, recognition by polyubiquitin-binding proteins, and recognition and degradation by the proteasome. The work presented in this thesis provides insight into the regulation of the polyubiquitination and the proteasome recognition steps, thus allowing a better understanding and a more complete picture of the p53 degradation pathway. The significance of this thesis work is that it may lead to the development of novel cancer therapeutics that target the p53 degradation pathway to cause p53 stabilization and

activation in tumors that retain wild type p53.

Based on this work, together with the previous knowledge of the p53 ubiquitination/degradation pathway, the following model for p53 degradation is proposed: 1) p53 is monoubiquitinated by the E3 ligase MDM2 causing its translocation from the nucleus to cytoplasm; 2) In the cytoplasm, CBP/p300 E4 ubiquitin ligase activity conjugates polyubiquitin chains onto monoubiquitimated p53; 3) polyubiquitinated p53 is next recognized by the hHR23 family of proteasome adaptor proteins, that then deliver p53 to the proteasome; 4) hHR23 proteins, in turn, are recognized by S5a, part of the 19S subunit of the proteasome, which may be the gateway to the proteasome for p53, leading to its destruction.

p53 polyubiquitination

Though p300 had been described in vitro as a p53 E4 (54), this work has demonstrated the physiological significance of CBP/p300 as E4 ubiquitin ligases of p53. Depletion of CBP or p300 resulted in p53 stabilization, and CBP/p300 were both required for physiologic p53 polyubiquitination. Moreover, a novel 2 step E4 assay allowed the separation of MDM2 from CBP confirming that CBP can act independently as an E4 without utilizing MDM2 as a catalytic cofactor to extend Ub chains.

Cytoplasmic compartmentalization

CBP and p300 E3/E4 ligase activities were only observed biochemically in cytoplasmic fractions. The spatial separation of CBP/p300's E3/E4 activity from their nuclear HAT functions solves the seeming paradox of how these two presumed opposing regulatory functions exist within the same molecules, but at the same time raising many intriguing questions.

First, what is the mechanism of compartmentalization of CBP/p300 E3/E4 ubiquitin ligase activity? Either a cytoplasmic-specific modification of CBP/p300 or CBP/p300 -interacting factor activates the activity or an interacting factor or modification in the nucleus represses it. The future direction for this part of the project would be to screen for the modification sites and type on CBP/p300 in nucleus vs. cytoplasm, or to discover interacting factors, which contribute to the activation or repression of CBP/p300 ligase activity.

Second, cytoplamic monoubiquitinated p53 is an active form to directly induce mitochondrial-mediated apoptosis upon stress via a transcription independent mechanism(105) . CBP/p300 cytoplasmic E4 activity obviously might repress this function of monoubiquitinated p53, converting the apoptotically active monoubiquitinated p53 to the unstable and inactive polyubiquitinated p53. Since we have shown that CBP/p300's E3/E4 activity is only active in cytoplasm in the absence of stress, it would be quite interesting to know how CBP/p300's E3/E4 activity is

regulated by DNA damage or other stress signals. We hypothesize that DNA damage signals may actively inhibit CBP/p300 E3/E4 activity in the cytoplasm, through direct modification or changes in cofactor binding to the E3 domain. This would allow accumulation of monoubiquitinated p53, which may remain in the cytoplasm and contribute to mitochondrial release of cytochrome C, or be imported into the nucleus to activate transcription.

Lastly, CBP/p300's E4 certainly could catalyze K48 polyubiquitin chain formation on p53 protein since CBP/p300 physiologically regulate p53 degradation. K63-linked p53-polyubquitin conjugates have also been observed in the cytoplasm but the function of these conjugates is not fully understood. Whether CBP/p300 cytoplasmic E3/E4 activity contributes in some way-negatively or positively-to K63-linked p53-polyubiquitin conjugates needs to be examined to gain a more complete understanding of how ubiquitin chain type and factors regulating Ub chain type impact p53 function.

Structure of the p300/CBP E3/E4 domain

p300/CBP lack an obvious canonical E3 domain, such as HECT, RING, PHD, or U-box. The N terminus of p300 harbors its E3/E4 activity. The same region of CBP encodes its E3/E4 activity and shares significant areas of sequence conservation with p300. The highly conserved C/H1 domain is included within the minimal region required for CBP/p300 E3 autoubiquitination activity, suggesting that the E3 activity

may center on this domain. This hypothesis is bolstered by modest similarity of C/H1 with other Zn2+ binding “atypical” E3’s, A20, Rabex-5, and E4F1 proteins (106). Of note, this C/H1 domain shares not even distant sequence similarity with RING, HECT, U-box, or PHD domains. The C/H1 domain will need to be carefully dissected for the minimal sequence necessary and sufficient for E3 activity by CBP/p300.

CBP catalyzes conjugation of polyubiquitin chains onto monoubiquitinated p53, but not native p53. The simplest explanation for the preferred recognition of a ubiquitinated substrate would be the existence of a Ub recognition (UBR) motif within the p300/CBP N-termini—as is seen in the Rabex-5 and Arf-BP1 E3 ligases. Besides the intrinsic E3 ligase domain, CBP/p300 E4 ligase activity might simultaneously rely on this UBR domain which can distinguish monoubiquitinated p53 from native p53, to perform its E4 ligase activity. Additionally, this UBR domain might even control the linkage type of polyubiquitin chain conjugated to p53.

The E4 ubiquitin ligase activity of CBP/p300 was separable from MDM2, but required MDM2 E3 monoubiquitination activity as a “priming” step. CBP/p300 and MDM2 coexist in one complex and their interaction is required for p53 degradation. Are CBP/p300 the generic E4 cooperating with all p53 E3s, or is CBP/p300 E4 ligase activity specific only to p53 that is monoubiquitinated by MDM2? Other E4s (besides YY1, which also utilizes MDM2 (138) for p53) might exist so that the specific E3-E4 couples might function under different conditions. The answer for these questions

remains unknown since all the work characterizing CBP/p300 E4 activity has been done in the context of MDM2 as the p53 E3. How the plethora of p53 E3s, and now E4s, are coordinated to achieve p53 homeostasis remains unclear.

The finding of CBP/p300 as p53 E4 ubiquitin ligases has potentially significant implications for cancer biology and therapy. MDM2 is now a bona fide clinically relevant drug target for cancer therapy. MDM2 inhibitors have been intensively investigated since they can activate p53 activity to kill cancer cells. Other enzymes in the p53 stability regulation pathway, such as the p300/CBP E4 domain, might also be effectively targeted in human cancers.

Proteasome recognition of p53

Upon polyubiquitination, p53 can face a number of fates, including degradation or deubiquitination to either an oligoubiquitinated form or an unconjugated form. HHR23 and related UBL-UBA proteins can act as proteasome adaptors regulating p53 degradation. In this thesis, I have shown that S5a, a 19S proteasome subunit, might be the entry point for hHR23 to the 26S proteasome to mediate p53 degradation.

S5a was required for p53 instability and also negatively influenced p53 activity. The specific role of S5a in unstressed p53 degradation is proposed to be as a substrate receptor for the proteasome, by recognizing either p53 directly, or via bridging by

hHR23 or other UBL-UBA proteins. In future experiments, this hypothesis will be tested in vivo by rescuing S5a-sh cells with wt and UIM mutant versions. Since the UIM mutant S5a is predicted to be defective in hHR23 binding, it should fail to rescue the inhibited p53 degradation in S5a depleted cells if hHR23 is the bridge for p53 delivering to S5a on the 26S proteasome. The study of S5a/hHR23 will not only delineate the final steps in the p53 degradation pathway, but also provide the general mechanism for protein substrate delivery into proteasome.

Role of the proteasome in p53 transactivation

Since S5a appeared to negatively regulate both p53 stability and p53 transactivation its role at a p53 target promoter was investigated. S5a was found coordinated with the p21 promoter pre-stress, but was lost from the promoter upon DNA damage, consistent correlating with possibly negative regulation of p53 target gene expression before and at some time after stress when the signal needs to be shut off. The clearance of transcription factors from promoters has been proposed as a chaperone-like non-proteolytic function of the 19S RP, of which S5a is a part (45). Given that S5a is a substrate receptor for degradation, we propose that it therefore is also the receptor for non-protease chaperone activities at active p53 promoters, perhaps via bridging by hHR23. The role of hHR23 and S5a in both degradation and transcription regulation indicates that the p53 ubiquitination/degradation and transcriptional

machinery might function coordinately.

In summary, p53 is under careful homeostatic regulation so that it is active only when and where it is needed. Understanding the spatial/temporal sequence of events in p53 degradation, and the specific biochemical roles of proteins (CBP/p300, S5a, hHR23) in each step in that process, is a prerequisite to understanding why the dysregulation of this pathway is selected for in certain cancers. The complete picture of this degradation pathway will aid us in designing effective therapeutics that can reactivate p53 in the cancer cells where the p53 degradation is dysregulated.

Differential regulation of p53 by p300 and CBP upon DNA damage

p300 and CREB-Binding Protein (CBP) act as multifunctional regulators of p53 via acetylase and ubiquitin ligase activities. The E3/E4 ubiquitin ligase activity is restricted within the cytoplasm, not interfering with CBP/p300 nuclear HAT activity. The physiologic role of the p300 and CBP coactivators in p53 regulation remains unclear, as most prior work has utilized overexpression approaches. The analysis of p300 or CBP-deficient tumor cells, p300 or CBP depleted untransformed MCF10A cells, and CBP (+/-, -/-) or p300 (+/-, -/-) MEFs revealed that CBP and p300 exhibited differential regulation of p53 biologic responses after DNA damage. CBP deficiency augmented, and p300 deficiency blocked, apoptosis, as well as PUMA transcription, after doxorubicin treatment. CBP and p300 seem to antagonize each other in regulating p53-induced apoptosis and apoptotic gene transcription.

Our results support the proposed transcription coactivator function of p300, at least when PUMA is the target gene, but is not consistent with a coactivation function for CBP. Conversely, CBP is more likely a corepressor of p53 on the PUMA promoter. Others have proposed that CBP is a p53 coavtivator based on analyses of the GADD45 and MDM2 promoters. One possibility for this inconsistency is that the property of each promoter determines coactivator or corepressor function of CBP (or p300). The underlying mechanism could be the recruitment of other proteins to form repressor or activator complexes with CBP (or p300) on specific promoters.

The stimulation of PUMA expression after CBP loss correlates with a loss of K320 acetylation. The mechanism by which p53 acetylation might negatively regulate p53 transactivation at specific genes is unknown and may include either or both recruitment of repressor complexes or the inability to recruit activating transcription factors. Additionally, acetylation is known to affect promoter-binding preference by p53, and this mechanism may also contribute to the observed effect of CBP deficiency and decreased K320 acetylation in increasing PUMA expression. Whatever the mechanism, the correlation of decreased K320 acetylation and increased PUMA expression and apoptosis has already been established in human cell lines and mouse knock-in systems (18).

As a factor that normally maintains p53 instability and suppresses apoptosis, CBP would be predicted to be maintained in tumors, and thus also represents a

potential therapeutic target for activating p53. p300, conversely, is mutated, albeit at low frequency, in certain human malignancies (50, 70) possibly due to the negative impact on the p53 pathway, and specifically p53-induced apoptosis. Given that cancer cells harboring wild-type p53 are specifically primed to undergo apoptosis when p53 is activated (14), mechanisms such as specific CBP inhibition may be of great value in cancer therapy, by increasing the therapeutic index of highly toxic chemotherapeutics such as doxorubicin.

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Appendix

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1 **Dingding Shi,** Marius S. Pop, Roman Kulikov, Ian M. Love, Andrew Kung, and Steven R. Grossman: CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proc Natl Acad Sci U S A, 106:16275-16280, 2009.

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